AD_____

Award Number: DAMD17-98-1-8490

TITLE: Herpes Virus Therapy of Prostate Cancer

PRINCIPAL INVESTIGATOR: Robert L. Martuza, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, Massachusetts 02114

REPORT DATE: August 2001

TYPE OF REPORT: Final, Phase I

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2001	Final, Phase I (1 Aug 98 - 31 Jul 01)

4. TITLE AND SUBTITLE
Herpes Virus Therapy of Prostate Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8490

6. AUTHOR(S)
Robert L. Martuza, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
Boston, Massachusetts 02114

E-Mail: martuza@partners.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The development of replication-competent viral vectors has offered a distinct advantage for prostate cancer gene therapy because of viral multiplication and spread within the tumor. G207, a second generation, multi-mutated HSV-1 vector has been shown to be very effective against human prostate cancer cell lines *in vitro* and *in vivo* following both direct intraneoplastic inoculation as well as following systemic intravenous inoculation. Moreover this efficacy applies to both androgen-sensitive as well as androgen-insensitive tumors and for post-irradiation recurrences. We now demonstrate that in a rodent prostate cancer model, NV1023 was more effective against Tramp tumors than either G207 or G47 delta, a G207 derivative. Following intraneoplastic therapy, the survival of NV1023 treated mice was extended by 5 weeks as compared to mock mice. Moreover in a bilateral tumor model , the virus treated (left) tumor volume was reduced to approximately one third the size of mock tumors. The untreated (right) tumors also regressed as expected with the tumor volume reduced to half the size of mock tumors.

We have completed our studies of G207 toxicity following intraprostatic inoculation in animal models and demonstrated that G207 can be safely inoculated into the prostate. We have also demonstrated that sequential rather than simultaneous irradiation may be as beneficial for vectors such as G207 in treating prostate cancer.

14. SUBJECT TERMS
Prostate Cancer

15. NUMBER OF PAGES
51

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

APPENDICES:

- Local and systemic therapy of human prostate adenocarcinoma with the conditionally replicating herpes simplex virus vector G207. Walker JR, McGeagh KG, Sundaresan P, Jorgensen TJ, Rabkin SD, Martuza RL. Human Gene therapy 10:2237-2243, 1999

- Pre-clinical safety evaluation of G207, a replication-competent herpes simplex virus type 1, inoculated intraprostatically in mice and nonhuman primates. Varghese S, Newsome JT, Rabkin SD, McGeagh K, Mahoney D, Nielsen P, Todo T, Martuza RL. Human Gene Therapy 12:999-1010, 2001.

- Ionizing radiation does not alter the antitumor activity of herpes simplex virus vector G207 in subcutaneous models of human and murine prostate cancer. Jorgensen TJ, Katz S, Varghese S, Rabkin SD, Martuza RL. (Submitted for publication)

INTRODUCTION: Our goal has been to develop oncolytic herpes vectors to treat prostate cancer and to acquire the necessary data to advance toward clinical trials. The development of replication-competent viral vectors has offered a distinct advantage for prostate cancer gene therapy because of viral multiplication and spread within the tumor. Replication-competent mutants of HSV-1 have been shown to be highly effective against a variety of experimental tumor models[1]. Oncolytic viral vectors based on engineered herpes simplex viruses offer unique advantages for prostate cancer therapy. They have minimal inherent immunogenicity and are much larger (153 kb) than those based on adenovirus or retrovirus backgrounds and thus can contain larger therapeutic gene elements. Safety features include well-identified genes for neurovirulence that can be deleted, and the availability of systemic anti-herpetic agents that can abort an unwanted infection. G207[2], a second generation, multi-mutated HSV-1 vector has been shown to be very effective against human prostate cancer cell lines *in vitro* and *in vivo* following both direct intraneoplastic inoculation as well as following systemic intravenous inoculation[3]. Moreover this efficacy applies to both androgen-sensitive as well as androgen-insensitive tumors and for post-irradiation recurrences.

BODY OF PROGRESS REPORT:

HYPOTHESIS 1: When inoculated intraneoplastically into a murine prostate cancer in an immune competent syngeneic mouse, HSV will induce a cell-mediated anti-tumor response in both the primary inoculated tumor as well as in distant metastases, and will prevent or delay the development of prostate cancer.

As noted in our prior progress report, herpes cytolytic activity in rodent cells is known to be far less efficient than in human cells and this delayed our work in murine syngeneic models to some extent. Moreover, the double gene mutations of G207 further attenuated replication in rodent cells. In Tramp mice (transgenic mice carrying the SV40 large T antigen gene under the control of the rat probasin promoter so that they spontaneously develop prostate tumors, which progressively metastasize)[4] we have begun to study other less attenuated HSV vectors. Tramp prostate cancer cell lines established from these mice grow well on syngeneic parental C57Bl/6 mice and thus provide for an immunocompetent model system to study both direct as well as immune mediated oncolytic effect. A direct comparison of the tumoricidal efficacy of G207, G47 delta and NV1023 was conducted using the Tramp prostate tumor model. Subcutaneous tumors generated by injecting 1 x 10^7 Tramp cells into the left flank of C57Bl/6 mice were allowed to grow until a diameter of 5-6 mm. At that time they were treated intratumorally twice, on days 0 and 3, with 1 x 10^7 pfu of either G207 or G47 delta or NV1023. Results, as shown in **Fig. 1A**, demonstrate that that NV1023 was more effective against Tramp tumors than either G207 or G47 delta. Survival of the treated mice was monitored until the tumor grew to a diameter of 21 mm, at which time the mice were sacrificed. As shown in **Fig. 1B,** the survival of NV1023 treated mice was extended by 5 weeks as compared to mock mice.

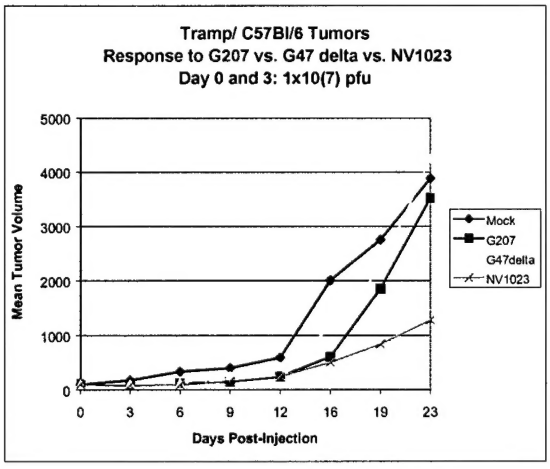

FIGURE 1A

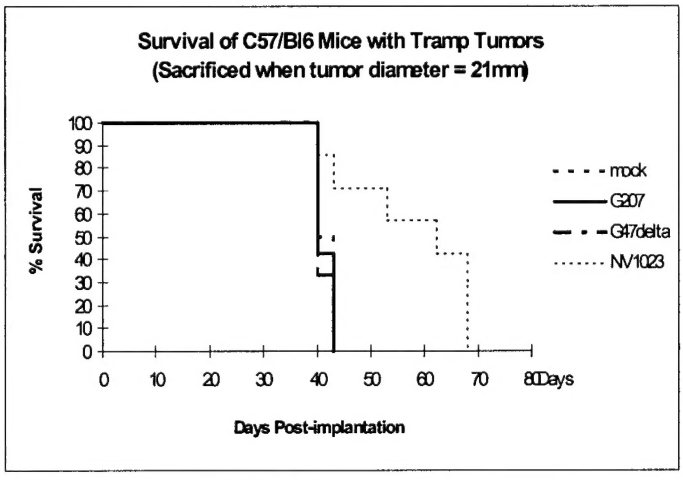

FIGURE 1B

G47 delta is derived from G207 and contains a 312 bp deletion in the US11 and ICP47 genes in addition to the deletions of both copies of ICP34.5 gene and a lacZ insertion inactivating the ICP6 gene. ICP47 gene product is associated with the down regulation of MHC class I molecules on the cell surface and therefore absence of this protein

provides for enhanced immune recognition of tumors[5]. NV1020 is an HSV1-HSV2 hybrid and retains one intact copy of the ICP34.5 gene. NV1023, derived from NV1020, has a deletion of the ICP47 gene similar to G47 delta. Since NV1023 has a deletion in ICP47 that renders an enhanced immunogenic effect, we tested the immune effect of NV1023 on contralateral Tramp tumors. For this, subcutaneous tumors were established on the left and right flanks of C57Bl/6 male mice by injecting 5 x 10^6 Tramp tumor cells on each flank. When the tumors reached 5-6 mm in diameter, the left tumor was treated with two inoculations of 1x 10^7 pfu of NV1023. By twenty days after treatment, the virus treated (left) tumor volume was reduced to approximately one third the size of mock tumors. The untreated (right) tumors also regressed as expected with the tumor volume reduced to half the size of mock tumors (**Fig. 3A and B**).

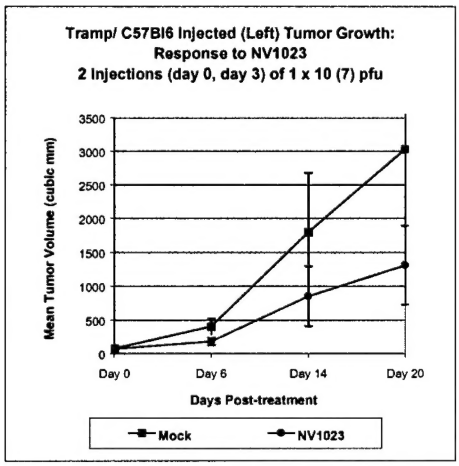

FIGURE 3A

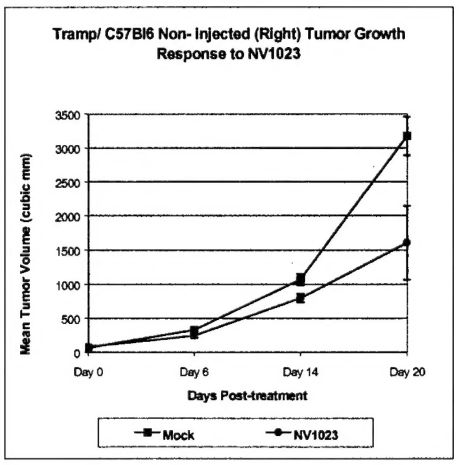

FIGURE 3B

TOXICITY OF INTRAPROSTATIC HSV (G207): We have completed our studies of G207 toxicity following intraprostatic inoculation and demonstrated in Balb/C mice (supported through this grant) and confirmed these results in primates (supported through other funding) that G207 can be safely inoculated into the prostate. ***A copy of this manuscript is enclosed which details these studies.***

HYPOTHESIS 2: As noted in the prior progress report, the initial funding letter noted that "This project was recommended for funding. The panel was intrigued with the possibility of using a mutated form of herpes virus as a novel therapy for prostate cancer but less enthusiastic about testing the IL-12 and GM-CSF vectors". Therefore, as noted in our prior progress report, we eliminated this hypothesis from the funded research of this initial grant and sought alternate funding for testing this hypothesis.

HYPOTHESIS 3: HSV VECTORS WILL SENSITIZE PROSTATE CANCERS TO RADIATION INDUCED CELL-DEATH IN VIVO.

It had been demonstrated at the time of this initial grant proposal that certain HSV mutants may be able to sensitize human brain tumor cells to radiation and that radiation could enhance viral replication. However, we could not confirm these results using prostate cancer cell lines in either athymic or in syngeneic animals. These findings are important in that they note previously unrecognized limitations on the work initially published by others and suggest that sequential rather than simultaneous irradiation may be as beneficial for vectors such as G207 in treating prostate cancer. ***A copy of this manuscript is enclosed which details these studies.***

KEY RESEARCH ACCOMPLISHMENTS:

- We have demonstrated that a conditionally-replicating HSV vector can kill or inhibit the growth of transgenic murine prostate cancer tumors in syngeneic animals
- We have demonstrated that G207 can effectively treat prostate cancers that have been previously irradiated but that simultaneous treatment with vector and irradiation is not synergistic.
- We have demonstrated prostate cancer killing can be made more effective by altering the genetic structure of the vector.
- We have demonstrated that the inoculation of one tumor can cause regression of a distant tumor, which in parallel studies by us has been demonstrated to be due to the induction of anti-tumor cell-mediated immunity.
- We have demonstrated in animal models that G207 can be safely inoculated into the prostate without toxicity forming the basis for a clinical trial for localized progressive prostate cancer following radiotherapy.

REPORTABLE OUTCOMES:

- Local and systemic therapy of human prostate adenocarcinoma with the conditionally replicating herpes simplex virus vector G207. Walker JR, McGeagh KG, Sundaresan P, Jorgensen TJ, Rabkin SD, Martuza RL. Human Gene therapy 10:2237-2243, 1999
- Pre-clinical safety evaluation of G207, a replication-competent herpes simplex virus type 1, inoculated intraprostatically in mice and nonhuman primates. Varghese S, Newsome JT, Rabkin SD, McGeagh K, Mahoney D, Nielsen P, Todo T, Martuza RL. Human Gene Therapy 12:999-1010, 2001.
- Ionizing radiation does not alter the antitumor activity of herpes simplex virus vector G207 in subcutaneous models of human and murine prostate cancer. Jorgensen TJ, Katz S, Varghese S, Rabkin SD, Martuza RL. Neoplasia, in press
- Favorable peer review and awarding of Phase II grant to continue this work "Herpes Virus Therapy of Prostate Cancer" (Aug1, 2001-Aug 31, 2003).

CONCLUSIONS:

We have demonstrated that human prostate cancers grown in athymic mice can be killed by genetically engineered conditionally replicating herpes viral vector (G207). Moreover, this has been demonstrated for both androgen-sensitive and androgen-insensitive tumors and for tumors recurring following irradiation. We next demonstrated that direct intraprostatic inoculation of the same vector (G207) did not cause toxicity in mice or in non-human primates. Just as our pre-clinical data in brain tumors formed the basis for the first clinical trial of G207 in glioma[6], taken together, these two enclosed manuscripts detail the preclinical data that forms the basis for a possible clinical trial in patients with

locally recurrent prostate cancer following irradiation.

We next demonstrated that improvements in vector design can treat murine prostate cancer in a syngeneic system and that inoculation of one tumor can induce an immune response against a distant tumor. We have termed this an "in situ vaccine" effect and we are developing this as a potential treatment for metastatic prostate cancer.

REFERENCES

1. Martuza RL. Conditionally-replicating herpes vectors for cancer therapy. J Clin Invest.105: 841-846, 2000

2. Mineta T, Yazaki T, Hunter W, Rabkin SD, Martuza RL. Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. Nature Medicine. 1:938-943, 1995.

3. Walker JR, McGeagh KG, Sundaresan P, Jorgensen TJ, Rabkin SD, Martuza RL.Local and systemic therapy of human prostate adenocarcinoma with the conditionally replicating herpes simplex virus vector G207. Human Gene therapy 10:2237-2243, 1999

4. Foster BA, Gingrich JR, Kwon ED, Madias C, Greenberg NM. Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer Research 57: 3325-3330, 1997

5. Todo T, Martuza RL, Rabkin SD, Johnson PA. Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. PNAS 98:6396-6401, 2001

6. Markert, JM, Medlock, MD, Rabkin, SD, Gillespie, GY, Todo, T., Hunter, WD, Palmer, CA, Feigenbaum, F., Tornatore, C., Tufaro, F. and Martuza, RL. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. Gene Therapy 7: 867-874, 2000

APPENDICES AND ATTACHMENTS:

- Local and systemic therapy of human prostate adenocarcinoma with the conditionally replicating herpes simplex virus vector G207. Walker JR, McGeagh KG, Sundaresan P, Jorgensen TJ, Rabkin SD, Martuza RL. Human Gene therapy 10:2237-2243, 1999

- Pre-clinical safety evaluation of G207, a replication-competent herpes simplex virus type 1, inoculated intraprostatically in mice and nonhuman primates. Varghese S, Newsome JT, Rabkin SD, McGeagh K, Mahoney D, Nielsen P, Todo T, Martuza RL. Human Gene Therapy 12:999-1010, 2001.

- Ionizing radiation does not alter the antitumor activity of herpes simplex virus vector G207 in subcutaneous models of human and murine prostate cancer. Jorgensen TJ, Katz S, Varghese S, Rabkin SD, Martuza RL. (Submitted for publication)

HUMAN GENE THERAPY 10:2237–2243 (September 1, 1999)
Mary Ann Liebert, Inc.

Local and Systemic Therapy of Human Prostate Adenocarcinoma with the Conditionally Replicating Herpes Simplex Virus Vector G207

JONATHAN R. WALKER,[1,2] KEVIN G. McGEAGH,[1,2] PERIASAMY SUNDARESAN,[1,3] TIMOTHY J. JORGENSEN,[4,5] SAMUEL D. RABKIN,[1,3,6] and ROBERT L. MARTUZA[1,3]

ABSTRACT

Prostate adenocarcinoma is the most common nonskin malignancy in males and the second most common cause of cancer death in the United States (Landis *et al.*, 1998). Initial treatments of surgery or radiotherapy may cause impotence and/or incontinence from neural damage (Eastham and Scardino, 1998; Porter *et al.*, 1998). When extraprostatic or metastatic disease develops, castration or pharmaceutical androgen ablation is utilized (Catalona, 1994). Androgen-resistant recurrence indicates a poor prognosis and justifies experimental chemotherapy (Oh and Kantoff, 1998). G207 (Mineta *et al.*, 1995; Yazaki *et al.*, 1995) is a multimutated herpes simplex virus 1 (HSV) vector that replicates within cancer cells, causing cellular death; however, replication is limited in normal cells, including those of the nervous system. *In vitro*, G207 at a low multiplicity of infection (MOI of 0.01) is oncolytic for multiple human prostate cancer cells. In athymic mice, a single intraneoplastic inoculation of G207 completely eradicates >22% of established subcutaneous human prostate cancer tumors irrespective of hormonal responsiveness. Two intraneoplastic inoculations of G207 completely eradicated two of three recurrent previously irradiated tumors and two intravenous administration of G207 induced tumor regression in distant subcutaneous tumors and completely eradicated one-fourth of the tumors.

OVERVIEW SUMMARY

G207 is a multimutated herpes simplex virus 1 vector that lacks both copies of the ICP34.5 gene and contains an insertion of the *lacZ* gene inactivating the ICP6 gene. G207 can replicate within cancer cells, causing cellular death; however, replication is limited in normal cells, including those of the nervous system. *In vitro*, at a low multiplicity of infection (MOI of 0.01), G207 is oncolytic for multiple human prostate cancer cell lines (LNCaP, DU-145, PC-3, and TSUPR-1). In athymic mice, a single intraneoplastic injection of G207 (2×10^7 PFU) into established subcutaneous human prostate cancer tumors caused a reduction in tumor volume (LNCaP, $p < 0.05$; DU-145, $p < 0.01$ versus controls) with complete eradication of >22% of tumors irrespective of hormonal responsiveness (LNCaP, 25%; DU-145, 22%). Two intraneoplastic injections of G207 (1×10^7 PFU) caused tumor volume reduction ($p < 0.05$ versus controls) and completely eradicated 66% of recurrent previously irradiated LNCaP tumors. Two intravenous doses of G207 (2×10^7 PFU) induced tumor regression in distant subcutaneous tumors (LNCaP, $p < 0.001$; DU-145, $p < 0.05$ versus controls) and completely eradicated one-fourth (LNCaP, 14%; and DU-145, 40%) of the tumors. G207 and related conditionally replicating HSV vectors warrant further evaluation for the treatment of local and metastatic prostate cancer.

INTRODUCTION

VARIOUS VIRAL VECTORS have been utilized for antiprostate cancer therapy (Eastham *et al.*, 1996; Rodriguez *et al.*, 1997; Hall *et al.*, 1998). However, delivery of viral particles to

[1]Molecular Neurosurgery Laboratory, [2]Department of Surgery (Division of Urology), [3]Department of Neurosurgery, [4]Department of Radiation Medicine, [5]Department of Biochemistry and Molecular Biology, and [6]Department of Microbiology and Immunology, Georgetown University Medical Center, Washington, D.C. 20007.

every cancer cell is a major limitation of this form of experimental therapy. Replication-competent vectors offer a potential advantage over replication-defective vectors owing to the possibility of viral multiplication within the tumor after viral delivery. The genetic modifications in G207 (deletion of both ICP34.5 genes and a *lacZ* insertion inactivating the ICP6 gene) permit replication within cancer cells but limit replication in normal cells, including those of the nervous system (Mineta *et al.*, 1995; Yazaki *et al.*, 1995). In athmic mouse models in which G207 has been studied, the antitumoral effect of G207 is primarily due to virally induced lysis of the infected cancer cells (Yazaki *et al.*, 1995; Toda *et al.*, 1998a). In immune-competent animals an additional benefit is due to the induction of an immune response to the tumor (Toda *et al.*, 1998b, 1999). For example, studies have shown tumor regression of noninoculated established subcutaneous tumors in syngeneic bilateral tumor models after intraneoplastic injection of G207 into a distant tumor. This effect was not due to systemic viral spread, but to the induction of tumor-specific cytotoxic lymphocytes (Toda *et al.*, 1999). This effect was lost when similar studies were done on immune-deficient animals.

G207 was originally developed to treat malignant brain tumors without harming surrounding normal brain cells (Mineta *et al.*, 1995; Yazaki *et al.*, 1995) and is now in a phase I clinical trial for recurrent malignant gliomas. More recently, G207 has been evaluated for the treatment of nonnervous system tumors (Toda *et al.*, 1998a, 1999). Because G207 is nonneurovirulent and neural damage is the major morbidity associated with the treatment of prostate cancer, G207 provides an alternative therapeutic vector for prostate cancer.

MATERIALS AND METHODS

Cell lines and in vitro *susceptibility testing*

Human-derived prostate cancer cell lines included LNCaP (provided by E.P. Gelmann, Georgetown University Medical Center, Washington, D.C.), DU-145 and PC-3 (provided by the Lombardi Cancer Center, Georgetown University Medical Center), and TSUPR-1 (provided by W. Isaacs, James Buchanan Brady Urological Institute, Baltimore, MD). LNCaP was main-

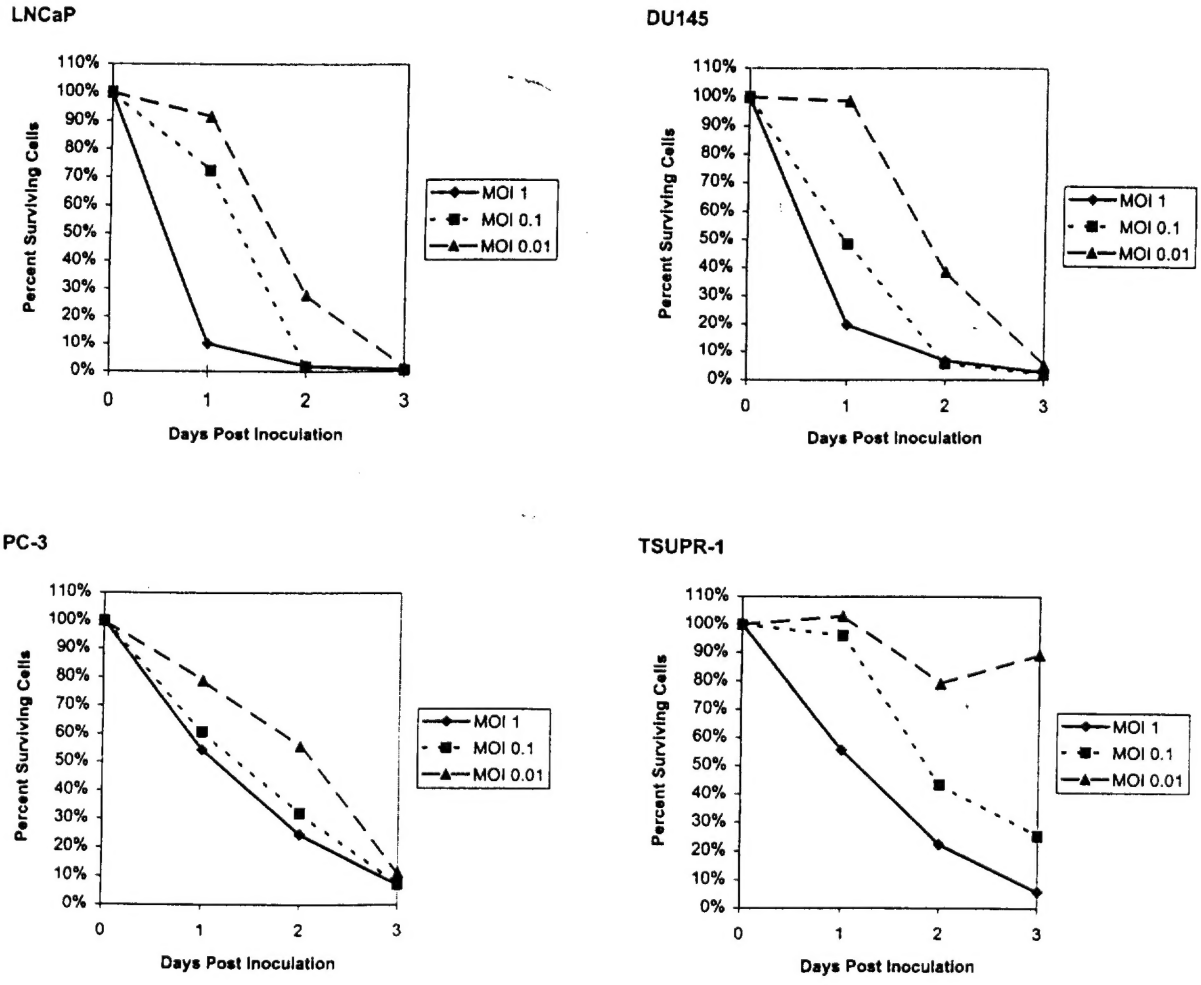

FIG. 1. G207 susceptibility of human-derived prostate cancer cell lines *in vitro*. Four human-derived prostate cancer cell lines (LNCaP, DU-145, PC-3, and TSUPR-1) were infected with G207 at MOIs of 1, 0.1, and 0.01. Each data point (mean of triplicate wells) is the percentage of surviving cells compared with the number of cells in control wells at each time point.

tained in Iscove's modified Eagle's medium (IMEM; Biofluids, Rockville, MD) containing 5% calf serum (HyClone, Logan, UT), DU-145 and PC-3 were maintained in RPMI 1640 (Biofluids) containing 10% fetal calf serum (HyClone), and TSUPR-1 was maintained in Dulbecco's modified Eagle's medium (Biofluids) with 10% fetal calf serum. All cell lines were maintained at 37°C in 5% CO_2 with penicillin and streptomycin (Sigma, St. Louis, MO) added to the medium, and were free of mycoplasma contamination. G207 (obtained from NeuroVir, Vancouver, BC, Canada) was titered on African green monkey (Vero) cells (provided by D. Knipe, Harvard Medical School, Boston, MA). For *in vitro* susceptibility assays LNCaP, DU-145, PC-3, or TSUPR-1 cells were plated in six-well dishes, infected with G207 at different multiplicities of infection (MOIs), and maintained in 1% heat-inactivated fetal calf serum in buffered saline at 34.5°C. Cell counts were done in triplicate using a ZM Coulter counter (Coulter, Miami, FL) for each MOI on days 1, 2, and 3 postinfection, after washing with phosphate-buffered saline (PBS) and detaching cells with trypsin–EDTA (GIBCO-BRL, Grand Island, NY).

Subcutaneous tumor model and intraneoplastic inoculation of subcutaneous tumors

Six- to 7-week-old male BALB/c *nu/nu* mice were obtained from the National Cancer Institute (Rockville, MD). The Georgetown University Animal Care and Use Committee approved all animal procedures. The mice were anesthetized with an intraperitoneal injection of a 0.25- to 0.30-ml solution consisting of 84% bacteriostatic saline (Abbott Laboratories, Chicago, IL), 10% sodium pentobarbital (1 mg/ml; Abbott Laboratories), and 6% ethyl alcohol or inhalation of 2–3 minimal alveolar concentration of methoxyflurane (Schering-Plough Animal Health, Union, NJ). Subcutaneous tumors were induced by flank injection of 5×10^6 LNCaP cells in 0.1 ml with an equal volume of Matrigel (Collaborative Biochemical Products, Bedford, MA), or 5×10^6 Du-145 cells in 0.1 ml. Tumors were measured by external caliper to within 0.1 mm, and volumes were calculated ($V = H \times L \times W$) and recorded. Animals with tumors less than 25 mm^3 were excluded. Tumors were inoculated intraneoplastically with G207 (2×10^7 PFU in 20 μl) or virus buffer (20 μl). Animals were sacrificed when the tumor diameter was greater than 18 mm.

Intraneoplastic inoculation in irradiated subcutaneous tumors

To study irradiated tumors, LNCaP tumors were established subcutaneously as described above, and animals randomized. Tumors received a total of 10, 20, 30, or 40 Gy fractionated over a 5-day period, using a cesium-137 irradiator or mock non-irradiated treatment. Tumors in the 10- and 20-Gy group recurred after initial radiation treatment. Animals with recurrent tumors were randomized into two new groups and the tumors were either inoculated intraneoplastically with G207 (1×10^7 PFU in 20 μl) or virus buffer (20 μl); repeat inoculation was done 2 days later.

Intravenous inoculation of G207

LNCaP or DU-145 tumors were established subcutaneously as described above, and randomized into two groups respectively. Animals were inoculated by tail vein with either G207 (2×10^7 PFU in 100 μl) or virus buffer (100 μl). This treatment was repeated 4 days later.

Serum PSA determination

At the completion of subcutaneous LNCaP tumor treatment, animals treated with either G207 (2×10^7 PFU) or virus buffer were sacrificed. Blood was obtained and centrifuged, and the

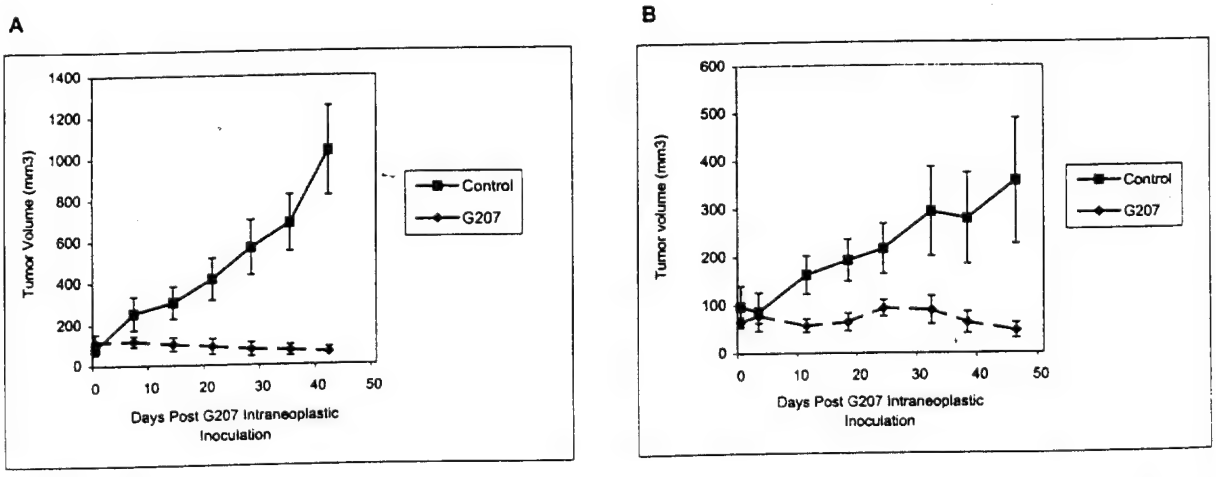

FIG. 2. The effect of intraneoplatic injection of G207 on subcutaneous human prostate tumor growth. (A) LNCaP and (B) DU-145 subcutaneous tumors were established in BALB/c *nu/nu* mice. When the mean volume of LNCaP tumors ($n = 8$) and DU-145 tumors (n = 1b) was 99 mm^3 (range, 28–224 mm^3) and 80 mm^3 (range, 26–350 mm^3), respectively, tumors received a single intraneoplastic injection of G207 (2×10^7 PFU) or virus buffer (day 0). G207-treated tumors showed a reduction in volume, whereas buffer-treated tumors continued to grow (LNCaP, $p < 0.05$ versus controls on day 42; DU-145, $p < 0.01$ versus controls on day 46). Complete eradication of 25 and 22% of the LNCaP and DU-145 tumors, respectively, was noted. (Bars represent mean tumor volume ± the standard error of the mean.)

serum was collected. Serum was sent to Bayer (Tarrytown, NY) for determination of total prostate-specific antigen (PSA). (PSA testing was kindly done by G. Allard.)

RESULTS AND DISCUSSION

The sensitivity of four human-derived prostate cancer cell lines (LNCaP, DU-145, PC-3, and TSUPR-1) to G207 was evaluated. Effective cytotoxicity, with >90% cell destruction within 3 days, was noted for three of four cell lines (LNCaP, DU-145, and PC-3) at an MOI of 0.01 (Fig. 1). This contrasts with studies of other viral vectors that require MOIs > 1 to achieve the same level of oncolysis for prostate cancer cell lines (Eastham *et al.*, 1996; Gotoh *et al.*, 1997). It also represents a higher level of oncolysis at lower MOIs than indicated in prior studies by us using G207 against human nervous system tumor cell lines (Yazaki *et al.*, 1995).

To study the effect of intraneoplastic G207 treatment on localized tumors, LNCaP and DU-145 were chosen for *in vivo* study since both cell lines had comparable *in vitro* oncolysis, while LNCaP is hormonally responsive and DU-145 is hormonally unresponsive (Tilley *et al.*, 1990). Hormonally unresponsive tumors are generally more aggressive, with an increased rate of metastasis and poorer patient survival (Stephenson *et al.*, 1992; Rembrink *et al.*, 1997; Oh and Kantoff, 1998). Established subcutaneous LNCaP ($n = 8$) or DU-145 ($n = 16$) tumors in athmic mice were inoculated with either G207 (2×10^7 PFU) or virus buffer intraneoplastically. Control tumor volumes increased to the point that the animals had to be sacrificed. In contrast to the controls, G207 induced significant tumor regression of LNCaP ($p < 0.05$ versus controls on day 42, t test) and DU-145 tumors ($p < 0.001$ versus controls on day 46, t test) (Fig. 2A and B). Treatment with one inoculation of G207 (2×10^7 PFU) resulted in eradication of 25% of the LNCaP tumors and 22% of the DU-145 tumors. LNCaP tumors produce human PSA, which can be measured in the serum. Mean total serum PSA was significantly lower (0.18 ng/ml) in the G207-treated group than in the virus buffer-treated group (102 ng/ml) ($p < 0.005$ versus controls, t test).

Because patients who choose radiation therapy as their primary treatment may develop a localized recurrence of prostate cancer the effectiveness of G207 on previously irradiated localized tumors was studied. Subcutaneous LNCaP ($n = 15$) tumors in athymic mice were initially irradiated. All tumors not irradiated (0 Gy) progressed until all animals had to be sacrificed. All tumors treated with 40 Gy and 33% of tumors treated with 30 Gy showed complete tumor eradication without recurrence. In contrast, all tumors treated with 10 or 20 Gy showed transient tumor volume reduction followed by regrowth. These 10- and 20-Gy radiation failures were then grouped together and randomized to receive either intraneoplastic injection of G207 (1×10^7 PFU) or virus buffer with treatment repeated 2 days later. All buffer-treated tumors continued to grow, whereas

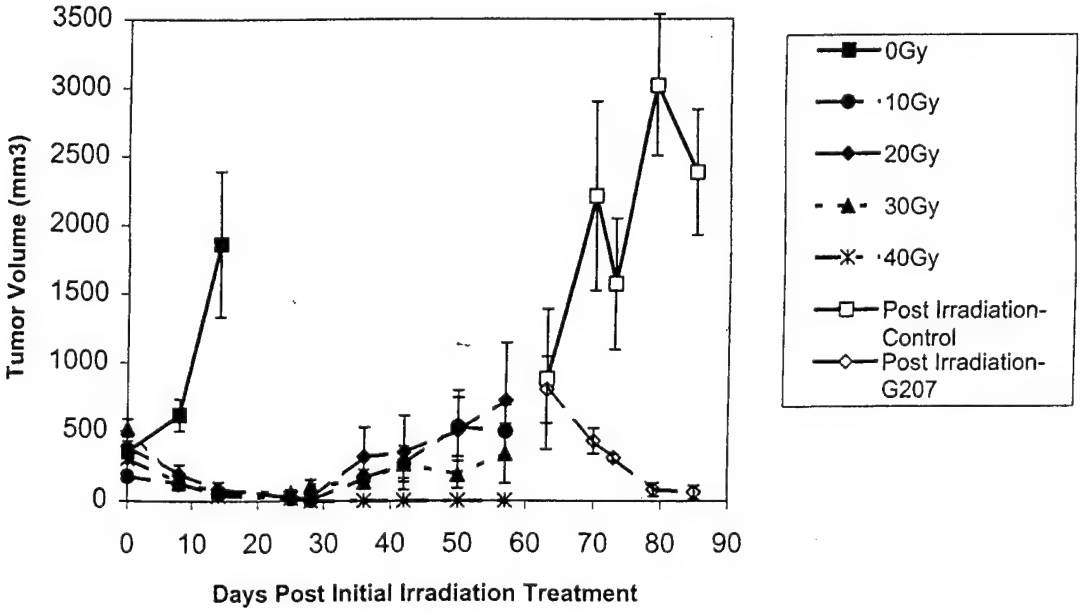

FIG. 3. The effect of radiation and G207 on subcutaneous LNCaP tumor growth. Subcutaneous LNCaP tumors were established in BALB/c *nu/nu* mice. When the mean tumor volume for LNCaP tumors ($n = 15$) was 348 mm^3 (range, 120–693 mm^3), tumors were randomized into five groups. Radiation was fractionated in equal doses over 5 days for a total dose of 10, 20, 30, or 40 Gy. Radiation treatment was noted to cause an initial reduction in tumor volume at all treatment doses when compared with 0 Gy. Tumor eradication was noted in 100% of the 40 Gy-treated tumors, and in 33% of the 30 Gy-treated tumors. All of these animals were then excluded from further studies. After the initial reduction in volume, all tumors in the 10- and 20-Gy treatment groups showed an increase in tumor volume. These tumors ($n = 6$) were then randomized to receive either G207 (1×10^7 PFU) intraneoplastically, followed 2 days later by a second injection, or two injections of virus buffer. G207-treated tumors had a marked reduction in tumor volume ($p < 0.05$ versus control on day 86) and tumor eradication was noted in 60% of the G207-treated tumors. (Bars represent mean tumor volume ± the standard error of the mean.)

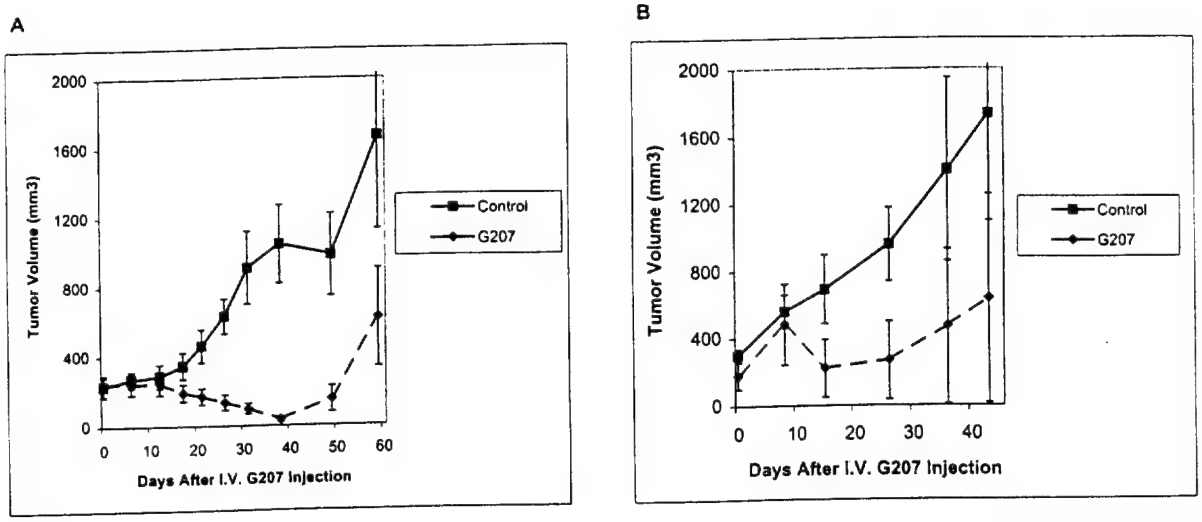

FIG. 4. The effect of intravenous injection of G207 on subcutaneous human prostate tumor growth. **(A)** LNCaP and **(B)** DU-145 subcutaneous tumors were established in BALB/c *nu/nu* mice. When the mean volume of LNCaP tumors ($n = 12$) and DU-145 tumors ($n = 12$) was 242 mm^3 (range, 96–385 mm^3) and 247 mm^3 (range, 63–510 mm^3), respectively, animals were injected intravenously (via tail vein) on day 0 and day 4 with G207 (2×10^7 PFU) or virus buffer. The mean tumor volume of G207-treated tumors showed a significant reduction versus buffer-treated tumors, which continued to grow (LNCaP, $p < 0.001$ versus controls on day 38; DU-145, $p < 0.05$ versus controls on day 26). Tumor regrowth was noted after the initial reduction in volume of both LNCaP and Du-145 tumors. Complete tumor eradication occurred in 14% (1 of 7) and 40% (2 of 5) of the LNCaP tumors and DU-145 tumors, respectively. (Bars represent mean tumor volume ± the standard error of the mean.)

two intraneoplastic treatments with G207 caused tumor eradication in 66% of these previously irradiated tumors ($p < 0.05$ versus controls on day 86, t test) (Fig. 3). While this total eradication rate is higher than that of Fig. 2, it should be noted that two additional variables must be considered. First, the animals in Fig. 3 received two doses of virus whereas those in Fig. 2 received only one dose. Second, experiments in other tumor systems have shown enhanced antitumor activity when radiation therapy is combined with oncolytic herpes simplex virus (HSV) therapy (Advani *et al.*, 1998). When this is done concomitantly increased viral growth has been documented. An effect seen after temporal spacing could be due to the interactions of apoptotic pathways, injury to tumor vasculature, or other mechanisms worthy of additional study. Whatever the mechanism, it is important to note that prior exposure to radiation did not negatively impact the effectiveness of G207. Thus further preclinical studies are warranted to consider viral oncolytic therapy for patients with recurrent prostate cancer after radiotherapy.

To test the possible use of intravenous delivery of G207 in the treatment of distant metastatic tumors, a preliminary study with subcutaneous DU-145 tumors was performed. DU-145 tumors ($n = 4$) were established subcutaneously in athymic mice and either G207 (2×10^7 PFU) or virus buffer was administered by tail vein inoculation. Animals were sacrificed on day 4, and tumors were removed and stained for β-galactosidase (Mineta *et al.*, 1994). Diffuse areas of LacZ expression were noted in the tumors of G207-treated animals but none was noted in the controls (data not shown). Therefore, LNCaP ($n = 12$) or DU-145 ($n = 12$) tumors were established subcutaneously in athymic mice. These mice were randomized and treated with either virus buffer or G207 (2×10^7 PFU) by tail vein inoculation. The treatment was repeated 4 days later. G207 caused significant tumor regression or growth inhibition versus virus buffer for both LNCaP ($p < 0.001$ versus controls on day 38, t test) and DU-145 ($p < 0.05$ versus controls on day 26, t test) (Fig. 4). Although inhibition or regression of tumor growth was observed with the dose regimen used, tumor recurrence was noted in the majority of the animals. Complete tumor eradication occurred overall in one-fourth of the tumors (one of seven LNCaP and two of five DU-145) treated with the two-dose regimen described.

This study has shown that intravenous delivery of G207 can induce tumor growth inhibition, regression, and eradication of distant prostate cancers. Others have demonstrated that localized vascular delivery of replicating HSV vectors is effective in the treatment of experimental brain tumors after intracarotid delivery (Rainov *et al.*, 1998) and in experimental hepatic tumors after hepatic artery delivery (Y. Fong, personal communication, 1999). A word of caution is in order since studies of wild-type and certain attentuated mutant strains of HSV have documented that intravenous administration can produce toxicity of spinal cord, adrenal glands, and liver (Hill *et al.*, 1986; Irie *et al.*, 1998). However, thus far, studies have shown that intravenous delivery of 10^7 PFU of G207 has been nontoxic in BALB/c mice (W. Hunter and P. Sundaresun, personal communication, 1999). Nonetheless, additional preclinical toxicity studies after both intravenous and intraprostatic G207 inoculation are warranted.

The mutations engineered into G207 are important for the significant reduction in neurovirulence after viral infection. G207 has been shown to be nonneurotoxic at 10^7 PFU after di-

rect inoculation into the brain of mice (BALB/c and AJ) (Mineta *et al.*, 1995), the sciatic nerve of mice (G. Mashour, personal communication, 1998), or into the brain of nonhuman primates (*Aotus*) (Mineta *et al.*, 1995). Further, G207 is currently in a phase I clinical trial for the treatment of recurrent malignant glioma and, as of this writing, 3×10^9 PFU has been inoculated intracranially into humans without the production of encephalitis or neural toxicity. Current techniques allow for well-localized stereotactic delivery of a vector to the prostate (Holm *et al.*, 1983). G207 could allow for the *in situ* ablation of prostate cancer cells in a "nerve-sparing" fashion, limiting the incontinence and impotence often associated with radiotherapy or surgery.

In conclusion, G207 may be valuable for treating various stages of prostate cancer including localized, hormonally resistant, previously irradiated, and metastatic tumors. Further improvement of these results may be possible simply by altering the dosing regimen as noted above. In addition, vector modifications providing improved viral replication, or the use of specific promoters, or the expression of cytokines and other anticancer genes, are worthy of development. Preclinical studies of possible toxicity after intravenous and intraprostatic inoculation will be necessary in order to consider this form of therapy for human use.

ACKNOWLEDGMENTS

We thank J.T. Newsome and A. Murray for their technical assistance with the project and veterinary support; P. Walsh, J. Lynch, E. Gelmann, and M. Lippman for inspiration and advice; G. Allard (Bayer Corp.) for the PSA determination; and P. Johnson (Neurovir, Inc.) for providing G207.

This work was supported in part by grants from CaPCURE and from the Department of Defense to R.L.M. (PRCP-97 IDEA).

S.D.R. and R.L.M. are consultants to Neurovir, Inc., which has a license from Georgetown University for G207.

REFERENCES

ADVANI, S.J., SIBLEY, G.S., SONG, P.Y., HALLAHAN, D.E., KATAOKA, Y., ROIZMAN, B., and WEICHSELBAUM, R.R. (1998). Enhancement of replication of genetically engineered herpes simplex viruses by ionizing radiation: A new paradigm for destruction of therapeutically intractable tumors. Gene Ther. **5,** 160–165.

CATALONA, W.J. (1994). Management of cancer of the prostate. N. Engl. J. Med. **331,** 996–1004.

EASTHAM, J.A., and SCARDINO, P.T. (1998). Radical prostatectomy. In *Campbell's Urology.* P.C. Walsh, A.B. Retik, E.D. Vaughan, and A.J. Wein, eds. (W.B. Saunders, Philadelphia, PA) pp. 2547–2564.

EASTHAM, J.A., CHEN, S.H., SEHGAL, I., YANG, G., TIMME, T.L., HALL, S.J., WOO, S.L., and THOMPSON, T.C. (1996). Prostate cancer gene therapy: Herpes simplex virus thymidine kinase gene transduction followed by ganciclovir in mouse and human prostate cancer models. Hum. Gene Ther. **7,** 515–523.

GOTOH, A., KAO, C., KO, S., HAMADA, K., LIU, T., and CHUNG, L. (1997). Cytotoxic effects of recombinant adenovirus p53 and cell cycle regulator genes (p21 WAF1/CIP1 and p16CDKN4) in human prostate cancers. J. Urol. 158, 636–641.

HALL, S.J., SANFORD, M.A., ATKINSON, G., and CHEN, S.H. (1998). Induction of potent antitumor natural killer cell activity by herpes simplex virus-thymidine kinase and ganciclovir therapy in an orthotopic mouse model of prostate cancer. Cancer Res. **58,** 3221–3225.

HILL, T.J., YIRRELL, D.L., and BLYTH, W.A. (1986). Infection of the adrenal gland as a route to the central nervous system after viraemia with herpes simplex virus in the mouse. J. Gen. Virol. **67,** 309–320.

HOLM, H.H., JUUL, H., PEDERSON, J.F., HANSEN, H., and STROYER, I. (1983). Transperineal I-125 seed implantation in prostatic cancer guided by transrectal ultrasonography. J. Urol. **130,** 283–286.

IRIE, H., KOYAMA, H., KUBO, H., FUKUDA, A., AITA, K., KOIKE, T., YOSHIMURA, A., YOSHIDA, T., SHIGA, J., and HILL, T. (1998). Herpes simplex virus hepatitis in macrophage-depleted mice: The role of massive, apoptotic cell death in pathogenesis. J. Gen. Virol. **79,** 1225–1231.

LANDIS, S.H., MURRAY, T., BOLDEN, S., and WINGO, P.A. (1998). Cancer statistics. Cancer J. Clin. **48,** 6–29.

MINETA, T., RABKIN, S.D., and MARTUZA, R.L. (1994). Treatment of malignant gliomas using ganciclovir-hypersensitive, ribonucleotide reductase-deficient herpes simplex viral mutant. Cancer Res. **54,** 3963–3966.

MINETA, T., RABKIN, S.D., YAZAKI, T., HUNTER, W.D., and MARTUZA, R.L. (1995). Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. Nature Med. **1,** 938–943.

OH, W.K., and KANTOFF, P.W. (1998). Management of hormone refractory prostate cancer: Current standards and future prospects. J. Urol. **160,** 1220–1129.

PORTER, A.T., LITTRUP, P., GRIGNON, D., FORMAN, J., and MONTIE, J.E. (1998). Radiotherapy and cryotherapy for prostate cancer. In *Campbell's Urology.* P.C. Walsh, A.B. Retik, E.D. Vaughan, and A.J. Wein, eds. (W.B. Saunders, Philadelphia, PA) pp. 2605–2626.

RAINOV, N.G., DOBBERSTEIN, K.U., HEIDECKE, V., DORANT, U., CHASE, M., KRAMM, C.M., CHIOCCA, E.A., and BREAKEFIELD, X.O. (1998). Long-term survival in a rodent brain tumor model by bradykinin-enhanced intraarterial delivery of a therapeutic herpes simplex virus vector. Cancer Gene Ther. **5,** 158–162.

REMBRINK, K., ROMIJN, J.C., VAN DER KWAST, T.H., RUBBEN, H., and SCHRODER, F.H. (1997). Orthotopic implantation of human prostate cancer cell lines: A clinically relevant animal model for metastatic prostate cancer. Prostate **31,** 168–174.

RODRIGUEZ, R., SCHUUR, E.R., LIM, H.Y., HENDERSON, G.A., SIMONS, J.W., and HENDERSON, D.R. (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: A selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. Cancer Res. **57,** 2559–2563.

STEPHENSON, R.A., DINNEY, C.P., GOHJI, K., OTDONEZ, N.G., KILLION, J.J., and FIDLER, I.J. (1992). Metastatic model for human prostate cancer using orthotopic implantation in nude mice. J. Natl. Cancer Inst. **84,** 951–957.

TILLEY, W.D., WILSON, C.M., MARCELLI, M., and McPHAUL, M.J. (1990). Androgen receptor gene expression in human prostate carcinoma cell lines. Cancer Res. **50,** 5382–5386.

TODA, M., RABKIN, S.D., and MARTUZA, R.L. (1998a). Treatment of human breast cancer in a brain metastatic model by G207, a replication-competent multimutated herpes simplex virus 1. Hum. Gene Ther. **9,** 2177–2185.

TODA, M., MARTUZA, R.L., KOJIMA, H., and RABKIN, S.D.

(1998b). In situ cancer vaccination: An IL-12 defective vector/replication-competent herpes simplex virus combination induces local and systemic antitumor activity. J. Immunol. **160,** 4457–4464.

TODA, M., RABKIN, S.D., KOJIMA, H., and MARTUZA, R.L. (1999). Herpes simplex virus as an "in situ cancer vaccine" for the induction of specific antitumor immunity. Hum. Gene Ther. **10,** 385–393.

YAZAKI, T., MANZ, H.J., RABKIN, S.D., and MARTUZA, R.L. (1995). Treatment of human malignant meningiomas by G207, a replication-competent multimutated herpes simplex virus 1. Cancer Res. **55,** 4752–4756.

Address reprint requests to:
Dr. Robert L. Martuza
Molecular Neurosurgery Laboratory
Georgetown University Medical Center
3800 Reservoir Road N.W.
Washington, D.C. 20007

E-mail: martuzrl@gunet.georgetown.edu

Received for publication March 15, 1999; accepted after revision June 17, 1999.

HUMAN GENE THERAPY 12:999–1010 (May 20, 2001)
Mary Ann Liebert, Inc.

Preclinical Safety Evaluation of G207, a Replication-Competent Herpes Simplex Virus Type 1, Inoculated Intraprostatically in Mice and Nonhuman Primates

SUSAN VARGHESE,[1] JOSEPH T. NEWSOME,[2] SAMUEL D. RABKIN,[1] KEVIN McGEAGH,[3] DREW MAHONEY,[3] PETUR NIELSEN,[4] TOMOKI TODO,[1] and ROBERT L. MARTUZA[1]

ABSTRACT

G207, a replication-competent herpes simplex virus type 1 (HSV-1) virus, has been previously shown to be effective against human prostate cancer xenografts in mice. This study assesses its safety in the prostate of two animal models known for their sensitivity to HSV-1. BALB/c mice were injected intraprostatically with either HSV-1 G207 or strain F and observed for 5 months. None of the G207-injected animals exhibited any clinical signs of disease or died. However, 50% of strain F-injected mice displayed sluggish, hunched behavior and died by day 13. Histopathologically, the G207-injected prostates were normal whereas strain F-injected prostates showed epithelial flattening, sloughing, and stromal edema. Four *Aotus nancymae* monkeys were also injected with G207 intraprostatically and observed short term (up to 21 days) and long term (56 days). Safety was assessed on the basis of clinical observations, viral biodistribution, virus shedding, and histopathology. None of the injected monkeys displayed evidence of clinical disease, shedding of infectious virus, or spread of the virus into other organs. Except for minor histological changes unrelated to the study, no significant abnormalities were observed. These results demonstrate that G207 can be safely inoculated into the prostate and should be considered for human trials for the treatment of prostate cancer.

OVERVIEW SUMMARY

G207, an attenuated replication-competent herpesvirus, offers distinct advantages for treating prostate cancer: it efficiently kills human prostate tumors in mice and, on the basis of safety studies in human brain and peripheral nerves in mice, it spares adjacent nervous system tissues. As part of the preclinical testing of G207 for treating prostate cancer, this study evaluated its safety in the prostates of BALB/c mice and *Aotus nancymae*. Results from morbidity, viral shedding, PCR-based detection of dissemination in various tissues, and histopathology of major tissues suggest that the virus can be safely inoculated into the prostate. Thus, the safety of G207 in prostate, coupled with its effectiveness in destroying human prostate cancers and its lack of neurovirulence, provides a strong basis for a human phase I clinical trial as a "nerve-sparing" method of treating prostate cancer.

INTRODUCTION

PROSTATE CANCER is the leading cause of cancer death among older men in Western countries and ranks second to lung cancer in cancer mortality (Woolf, 1995). The incidence of prostate cancer has increased steadily in the last two decades and is the most frequently diagnosed noncutaneous neoplasm. The availability of prostate-specific antigen (PSA) as a tumor marker has profoundly enhanced the early detection and diagnosis of clinically significant prostate cancer (Potosky *et al.*,

[1]Molecular Neurosurgery Laboratory, Department of Surgery (Neurosurgery), Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129.
[2]Department of Pathology and Division of Laboratory Animal Resources, University of Pittsburgh, Pittsburgh, PA 15261.
[3]Department of Urology, Georgetown University Hospital, Washington, D.C. 20007.
[4]Department of Pathology, Massachusetts General Hospital, Boston, MA 02114.

1995). Nevertheless, substantial controversy surrounds the most appropriate treatment.

Early-stage, organ-confined tumor is usually treated by radical prostatectomy or radiation therapy (external beam or brachytherapy). Locally advanced cancers or metastatic disease, with lymph node, lung, or skeletal bone involvement, are treated by ablative androgen therapy and/or radiation therapy. However, with time, androgen-independent disease resistant to the standard therapeutic regimen may arise (Dorkin and Neal, 1997). Moreover, present treatment strategies (radical prostatectomy, external beam irradiation, and brachytherapy) may be associated with risks of urinary incontinence, proctitis, or erectile dysfunction.

Many features unique to prostate cancer make it an ideal candidate for gene therapy; prostate cancer is relatively slow growing, the gland is easily accessible, and a circulating marker, PSA, is available for early detection and for monitoring the treatment response. A serious limitation of gene therapy protocols is the lack of efficient delivery of the vector to every cancer cell (Peng and Russell, 1999). Until recently, various gene therapy studies against prostate cancer have utilized replication-deficient adenoviruses (Eastham *et al.*, 1996; Ko *et al.*, 1996; Herman *et al.*, 1999; Lu *et al.*, 1999; Nasu *et al.*, 1999). The development of replication-competent viral vectors (adeno, herpes, and vaccinia viruses) has offered a distinct advantage of viral multiplication and spread within the entire tumor. Replication-competent adenoviruses CN706 (Rodriguez *et al.*, 1997), and CV764 and CV787 (Yu *et al.*, 1999a,b), constructed with specificity to the prostate have been shown to be effective against prostate cancer cells and tumor xenografts. However, both adenovirus and vaccinia virus vectors elicit strong host immune responses that may limit their efficacy. This shortfall may be overcome by the use of herpesvirus vectors. Attenuated replication-competent mutants of herpes simplex virus type 1 (HSV-1) have been shown to be highly effective against a variety of experimental tumor models (Martuza *et al.*, 1991; Boviatsis *et al.*, 1994; Mineta *et al.*, 1994; Yazaki *et al.*, 1995; Andreansky *et al.*, 1996; Brandt *et al.*, 1997; Kramm *et al.*, 1997; Pyles *et al.*, 1997; Randazzo *et al.*, 1997; McKie *et al.*, 1998; Yoon *et al.*, 1998; Lambright *et al.*, 1999; Toyoizumi *et al.*, 1999).

G207 is a second-generation, multimutated HSV-1 vector that was initially developed as a therapeutic agent for brain tumors with the specific aim of targeting cancer cells but not normal cells within the brain. The virus has been genetically altered, through deletion of both copies of the γ34.5 (RL1) gene and an *Escherichia coli lacZ* insertion inactivating the ICP6 gene, in order to permit replication within cancer cells but not in normal cells (Mineta *et al.*, 1995). Moreover, the virus is hypersensitive to the antiviral drugs ganciclovir and acyclovir. G207 has been shown to be effective against many different types of tumors, including malignant glioma, meningioma and neuroblastoma, gastric cancer, cancers of the prostate, bladder, breast, colon, pancreas, ovaries, and head and neck (Mineta *et al.*, 1995; Yazaki *et al.*, 1995; Toda *et al.*, 1998; Chahlavi *et al.*, 1999; Lee *et al.*, 1999; Walker *et al.*, 1999; Bennett *et al.*, 2000; Coukos *et al.*, 2000; Oyama *et al.*, 2000).

The significance of using G207 in treating human prostate cancer *in vivo* is based on the following two observations: (1) the virus demonstrates effective oncolysis of human prostate cancer cell lines *in vitro* and in *in vivo* tumor models established in mouse (Walker *et al.*, 1999); and (2) safety evaluation of the virus injected intracerebrally in HSV-1-sensitive nonhuman primates (*Aotus nancymae*) and via intracerebral, intracerebroventricular, intravenous, or intrahepatic routes in mice demonstrated no HSV-associated neural or general pathology or disease (Hunter *et al.*, 1999; Sundaresan *et al.*, 2000; Todo *et al.*, 2000). G207 was also demonstrated to be safe in humans, in a phase I clinical trial of patients with malignant gliomas (Markert *et al.*, 2000). Direct stereotactic intratumoral inoculation of up to 3×10^9 PFU was tolerated without the production of encephalitis or other limiting toxicity. Furthermore, it has been shown that injection of G207 into healthy sciatic nerves in BALB/c mice caused no neurological sequelae (Mashour *et al.*, 2001). These studies suggest that G207 may be a promising agent for prostate cancer therapy by destroying tumor cells without harming adjacent nerves. Targeted delivery of G207 or other replicating viral vectors is currently possible because of the availability of precise stereotactic injection of a vector into the prostate (Holm *et al.*, 1983).

This study was aimed at evaluating the safety of G207 injected intraprostatically in mice and New World owl monkeys (*Aotus nancymae*). The results, based on observations of morbidity, mortality, biodistribution (based on polymerase chain reaction [PCR] detection of viral DNA), and histopathology, suggest that the virus can be safely inoculated into the prostate. When considered in conjunction with prior studies of efficacy in treating prostate cancer (Walker *et al.*, 1999), the current study strongly suggests that G207 be considered in human trials as a nerve-sparing treatment for prostate cancer.

MATERIALS AND METHODS

Viruses

G207 (Mineta *et al.*, 1995) was obtained from NeuroVir (Vancouver, BC, Canada), and viral stocks were prepared as described previously (Todo *et al.*, 2000). HSV-1 strain F was provided by B. Roizman (University of Chicago, Chicago, IL). Viruses were titered on Vero (African Green monkey kidney) cells as previously described (Miyatake *et al.*, 1997).

Animals

Six- to 7-week-old BALB/c male mice were obtained from the National Cancer Institute (Frederick, MD). New World owl monkeys (*Aotus nancymae*) were obtained through the Veterinary Resources Program, National Center for Research Resources, National Institutes of Health (Poolesville, MD). The six monkeys used for the study were between 4 and 8 years of age and weighed approximately 840–1264 g. Monkeys T348 and T442 had received two intracerebral (left frontal lobe) inoculations of G207 (Hunter *et al.*, 1999) 2 and 3 years prior to the start of this study whereas T635 and T659 were naive for known exposure to HSV. Two other preexposed (prior intracerebral inoculation) animals, T317 and T344 (Hunter *et al.*, 1999), were used as prostate-noninjected controls. All animal studies were conducted at Georgetown University under the review and approval of the Georgetown Animal Care and Use

Committee consistent with Public Health Service policies as outlined in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, National Academy Press, Washington, D.C., 1996).

Intraprostatic injection of mice and monkeys

Mice were anesthetized via inhalation of methoxyfluorane (Schering-Plough Animal Health Corporation, Union, NJ). The abdominal hair was shaved and the skin was disinfected with povidone-iodine (Betadine) and 70% alcohol. A midline skin incision was made in the lower abdomen and the abdominal wall was opened between the rectus muscles. The urinary bladder and seminal vesicles were identified and retracted so as to expose the dorsolateral prostate. Twenty microliters of G207 (1×10^7 PFU), strain F (1×10^6 PFU), or virus buffer consisting of sterile 10% glycerol in phosphate-buffered saline (mock) was injected with the aid of a dissecting microscope into the left lobe of the prostate, using a 27-gauge needle. A bleb beneath the prostate capsule confirmed the injection. The abdominal contents were repositioned, the fascia was closed with absorbable sutures, and the skin was reapproximated with wound clips.

Monkeys were sedated with tiletamine plus zolazepam (Telazol, 4 mg/kg; A.H. Robbins, Richmond, VA). A 24-gauge angiocast was placed in the lateral saphenous vein and anesthesia was induced with a bolus of propofol (Diprivan, 40–60 mg; Astra Zeneca, Wilmington, DE). Monkey maintenance anesthesia was with an intravenous drip infusion (50–100 ml/kg/hr) of propofol. A linear midline incision was placed in the lower abdominal region to expose the urogenital organs. G207 (1×10^7 PFU) in 20 μl was injected with a 27-gauge needle into the left lobe of the prostate. The body wall was closed with Vicryl sutures in a simple interrupted pattern and skin was closed using a subcuticular pattern.

Clinical monitoring

After inoculation of the viruses into the prostate, the animals were closely monitored for any behavioral changes. The monkeys underwent regular physical examinations that included periodic temperature and body weight determinations and neurological assessment. Blood analysis, including serum clinical chemistries and complete blood cell counts, was done at 7- or 14-day intervals for the short-term and long-term animals, respectively. Serum anti-HSV-1 antibody for each of the monkeys was determined pre- and postinjection, using an enzyme-linked immunosorbent assay (ELISA) by BioReliance Corporation (Rockville, MD). The animals were killed at designated time points: the short-term monkeys T348 and T635 on days 14 and 21, respectively, and long-term monkeys T442 and T659 on day 56. For each animal, all the tissues, including the prostate (approximately 0.7×0.25 cm in size per lobe), were divided into three pieces: one each for PCR, histopathology, and frozen storage.

Virus shedding studies

Blood, cerebrospinal fluid (CSF), urine, and mucosal swabs of urethral, rectal, buccal, and conjuctival sites were collected on days 7, 14, and 21 for the short-term monkeys and on days 14, 28, and 56 for the long-term monkeys. The sterile sample swab tip was placed in 2 ml of Multi-Microbe medium (M-4; Micro Test, Snellville, GA), snap frozen, and stored at −80°C.

Shedding of infectious virus in each sample was determined by culturing the samples over a monolayer of Vero cells (conducted by NeuroVir). Vero cells plated in a 6-well plate at a density of 2×10^5 cells/well were incubated for 4 hr at 37°C to allow cell attachment. Sample was then added at 1:2 or 1:10 dilution in 1 ml and allowed to incubate overnight to aid viral adsorption. Another 1 ml of growth medium containing 10% fetal calf serum in Dulbecco's minimum essential medium was added and the cells were observed daily for 7 days for cytopathic effect.

Biodistribution studies by PCR analysis

Tissue distribution studies were conducted by Bioreliance, using a standardized real-time (TaqMan) PCR assay. Detection of HSV-1 DNA polymerase sequences was done with total DNA isolated from tissues collected at necropsy. The assay was consistently linear between 10 and 10^5 template copies and detects 100 copies of template in the presence of 1 μg of monkey genomic DNA.

Histopathology

The samples for histopathology were sent to Pathological Associates International (PAI, Frederick, MD) for analysis. Tissues obtained at biopsy were fixed in formalin, embedded in paraffin, sectioned on a sliding microtome (8 μm thick), and stained with hematoxylin and eosin. The histopathology slides were blinded and reviewed independently by two board-certified pathologists: one of the authors (P.N.) and the other from PAI.

RESULTS

The intraprostatic safety of G207 was tested in two different HSV-susceptible animal models: mice (BALB/c) and nonhuman primates, *Aotus nancymae*.

Intraprostatic injection of HSV in BALB/c mice

Clinical observations and survival. BALB/c mice received intraprostatic inoculation of G207 (1×10^7 PFU), wild-type strain F (1×10^6 PFU), or mock ($n = 8$, 8, and 14, respectively). Two animals from the mock-injected group died within the 48-hr post-operative period and these deaths were ascribed to anesthesia and surgery. The surviving animals were observed daily for the first 14 days and thereafter periodically for any changes in behavior or activity. There was no obvious change in activity or behavior in any of the G207- or mock-injected animals. All eight of the G207-injected animals survived until 5 months postinjection, when they were killed. Only the HSV-1 wild-type strain F-injected mice exhibited any clinical changes. Their behavior was sluggish in the first few days postinjection and four of eight animals also had hunched posture. All of these hunched animals died by day 13 (Fig. 1).

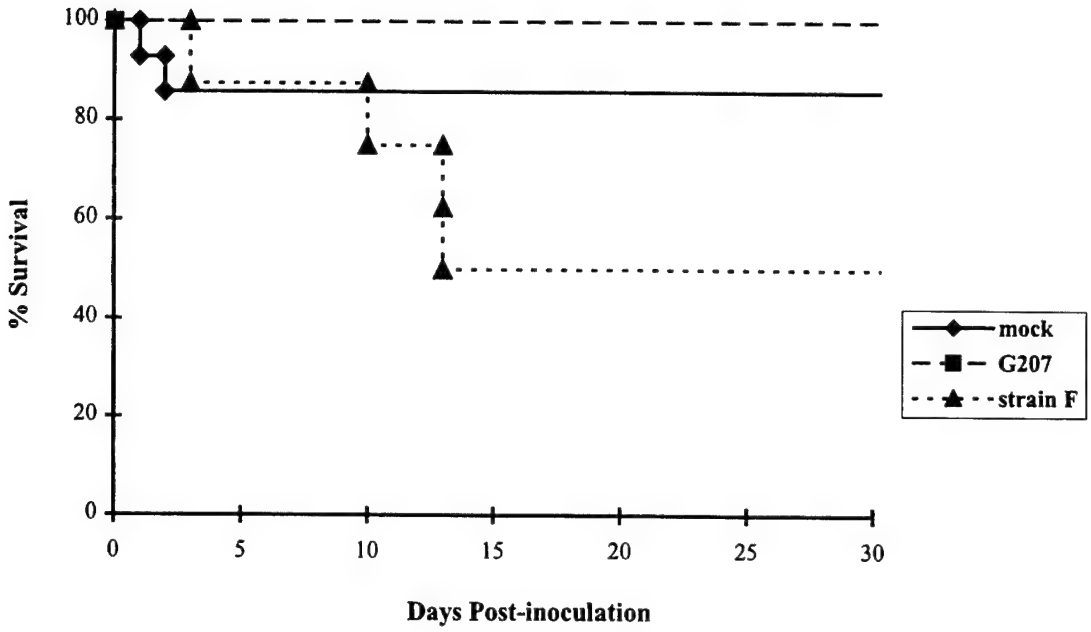

FIG. 1. Survival of BALB/c mice after intraprostatic inoculation with HSV. Eight mice each were injected with either 1×10^7 PFU of G207 or 1×10^6 PFU of strain F in a 20-μl volume and another 14 mice (mock) were injected with 20 μl of virus buffer. The animals were observed for 5 months; data shown here are for the first 30 days after injection, as there was no change in status in the subsequent months.

Histopathology. Prostate tissues were obtained at necropsy and histological analysis was performed by hematoxylin and eosin staining (Table 1 and Fig. 2). None of the G207- or mock-injected BALB/c mice showed any abnormal pathology (Fig. 2, top), whereas all the strain F-injected mice exhibited varying degrees of epithelial sloughing and stromal edema in the prostate tissue (Fig. 2, bottom). In one injected mouse (F-4) from the strain F group, only minimal prostate was detected and in another mouse (F-1), no prostate tissue could be identified although normal fat was observed (Table 1).

Intraprostatic injection of G207 in nonhuman primate (Aotus nancymae)

The G207 safety study in *Aotus nancymae* consisted of a short-term arm and a long-term arm, with two animals per arm. Each arm consisted of one previously inoculated animal (T348 and T442) and one naive animal (T635 and T659). The short-term animals, T348 and T635, were sacrificed on days 14 and 21, respectively, after intraprostatic injection of G207 while the long-term animals, T442 and T659, were sacrificed on day 56. Two monkeys, T317 and T344, included as prostate-noninjected controls, were sacrificed to compare the normal histology of prostates in these animals. Because of the known toxicity of the wild-type strain F in *Aotus* (Meignier *et al.*, 1990; Hunter *et al.*, 1999) and the demonstrated toxicity of intraprostatic injection of strain F in BALB/c mice (Fig. 1), no strain F virus was inoculated into monkeys.

Clinical observations and survival. Clinical monitoring, including activity and behavior checks, was performed on a daily basis. Body weight and temperature were measured at regular intervals until the time of sacrifice. There was no significant change in body weight or temperature during the course of the study, in any of the monkeys. None of the monkeys exhibited any altered behavior or died during the period of the study, and they were killed at the designated time points.

Blood was collected at 7- or 14-day intervals and analyzed for complete blood count (CBC), differential count, and serum chemistry (Table 2). The study subjects showed some variations from the normal range calculated from the preopation values of the four prostate-injected (T348, T442, T635, and T659) and the two prostate-noninjected (T317 and T344) animals. The prostate-injected monkeys were also tested for the presence of HSV antibody by ELISA from serum samples obtained pre- and postintraprostatic injection with G207 (Table 3). T348 and T442 had a positive titer both prior to and after the intraprostatic injection. Monkey T659 was naive at the onset of the study and, as expected, seroconverted after G207 injection into the prostate.

Virus shedding in Aotus. To determine whether intraprostatic injection would lead to virus shedding, urine, serum, and swabs from various mucosal regions (buccal, conjunctiva, urethra, and rectum) were tested for the presence of infectious virus by plaque assay. No virus was detected in any of these samples (data not shown). We attempted to collect semen samples by electroejaculation but were unsuccessful in most cases except for T442, from which semen sample was obtained on days 0 and 14, and no virus was detected from these samples. CSF was collected at necropsy from the long-term monkeys only and was negative for infectious virus.

TABLE 1. HISTOLOGY OF PROSTATE TISSUE FROM BALB/c MICE INOCULATED INTRAPROSTATICALLY WITH HSV[a]

Mock			*G207*			*Strain F*		
Mouse	*Survival day (D or S)*	*Histology*	*Mouse*	*Survival day (D or S)*	*Histology*	*Mouse*	*Survival day (D or S)*	*Histology*
M-1	151 (S)	Normal	G-1	151 (S)	Normal	F-1	158 (S)	No prostate detectable; normal fat
M-2	151 (S)	Normal	G-2	151 (S)	Normal	F-2	158 (S)	Stromal edema, some epithelial flattening
M-3	151 (S)	Normal	G-3	151 (S)	Normal	F-3	13 (D)	Sloughed epithelial cells, stromal edema
M-4	151 (S)	Normal	G-4	151 (S)	Normal	F-4	13 (D)	Minimum prostate detected with sloughed epithelium and stromal edema
M-5	151 (S)	Normal	G-5	151 (S)	Normal			
M-6	151 (S)	Normal	G-6	151 (S)	Normal	F-5	3 (D)	Epithelial (focal) flattening and sloughing
M-7	158 (S)	Normal	G-7	151 (S)	Normal	F-6	10 (D)	Epithelial sloughing and stromal edema
M-8	158 (S)	Normal	G-8	151 (S)	Normal	F-7	120 (D)	NA
M-9	158 (S)	Normal				F-8	152 (D)	NA
M-10	158 (S)	Normal						
M-11	158 (S)	Normal						
M-12	158 (S)	Normal						

[a]Histology of prostate tissue from BALB/c mice inoculated intraprostatically with HSV. Mock mice were injected with 20 μl of virus buffer; G207 mice and strain F mice received either 1×10^7 or 1×10^6 PFU, respectively, in a 20-μl volume. Tissues were collected at necropsy, fixed in formalin, and paraffin embedded. Sections were stained with hematoxylin and eosin. D, death; S, sacrifice; NA, tissue not available (F-7 and F-8 died secondary to cagemate trauma).

Virus biodistribution in Aotus. To determine the distribution of intraprostatically injected G207 to other organs, tissue samples from necropsy were analyzed by real-time quantitative PCR for HSV DNA polymerase sequences. High copy numbers of HSV DNA were detected in the brain (temporal and frontal lobe) and spinal cord of the preexposed (intracerebrally injected) animals, T348 and T442 (Table 4). One long-term animal, T659, had quantifiable but low copy numbers of HSV DNA present in the local retroperitoneal lymph node (12 copies), spleen (28 copies), and urethra (penile, 66 copies). Nonquantifiable amounts (1–10 copies) of DNA were detected in a few tissues, including spinal cord (T348), retroperitoneal ganglia (T659), retroperitoneal lymph node (T348 and T635), adrenals (T635), spleen (T442), and prostate (T659).

Histopathology of Aotus. At necropsy, the major tissues from each of the monkeys were weighed and dissected to check for gross morphology. No significant abnormalities were seen in any of the organs in these animals, except for the prostate of T659, which exhibited surgery-related scarring in the left lobe (injection site). Histopathological examination revealed no abnormalities in the prostate of T635 and T442 (Fig. 3 and Table 5), or in the erectile tissues of T348, T442, and T635. Prostate tissue was not identifiable in the formalin-fixed wet tissues from T348 and T659, although prostates with normal morphology were seen in these monkeys at the time of necropsy. Most likely, for T348 and T659, the majority of the prostate (approximately 0.7×0.25 cm in size per lobe) was used for PCR and only periprostatic tissue was found in the formalin-fixed sample. These adjuvant tissues were found to have normal histology. However, the possibility that G207 could destroy some normal prostate tissue cannot be excluded.

All animals, including the prostate-noninjected T317 and T344 monkeys, exhibited interstitial inflammation in the kidney, and abnormalities in lung (mild vascular congestion) and liver (Kupffer cell hemosiderin pigmentation, prominent lipofuscin in hepatocyte, centroacinar dissociation, degeneration of hepatocytes) (Table 5). These changes are common observations in *Aotus nancymae* (Todo *et al.*, 2000).

DISCUSSION

Previous studies from our laboratory have demonstrated that G207 is effective in killing human prostate cancer cells (Walker *et al.*, 1999). Both androgen-dependent (LNCaP) and androgen-independent (DU-145, PC-3, and TSUPR-1) cell lines were sensitive to G207 (Walker *et al.*, 1999). This observation is important in light of the common development of androgen-refractory disease during the natural course of prostate cancer progression. In addition, we have also shown that subcutaneous human prostate cancer tumors generated in athymic or immunocompetent mice respond efficiently to G207 administered either intratumorally or intravenously (Walker *et al.*, 1999; Jorgensen *et al.*, 2001). Thus, G207 would be an ideal vector candidate to be used in viral therapy studies to treat prostate cancer.

As part of the preclinical testing of G207, the current study was aimed at assessing the safety of intraprostatic injection of G207 in two animal models known for their sensitivity to HSV-1: BALB/c mice and a nonhuman primate, *Aotus nancymae*. We have demonstrated that direct injection of G207 into the brain of these animals or via intracerebroventricular, intravenous, and intrahepatic routes in BALB/c mice did not result in any toxicity or complications (Hunter *et al.*, 1999; Sundaresan *et al.*, 2000; Todo *et al.*, 2000). G207 dose of up to 3×10^7 PFU in mice and up to 1×10^9 PFU in monkeys could be administered safely whereas 1×10^3 PFU of the wild-type strain F virus resulted in pathology in these animals. Extensive histopathological and PCR studies did not reveal any evidence

TABLE 2. BLOOD ANALYSIS OF *Aotus nancymae* INOCULATED INTRAPROSTATICALLY WITH G207[a]

Analyte	*Preoperation average*	*T348 (PI day 7)*	*T635 (PI day 14)*	*T442 (PI day 14)*	*T659 (PI day 14)*
Glucose, mg/dl	93–165	159	162	93	184
Urea nitrogen, mg/dl	15–23	21	18	30	20
Creatinine, mg/dl	0.9–1.1	1.2	0.9	1.2	1.4
Total protein, g/dl	7.7–8.3	9	6.9	8.4	8.6
Albumin, g/dl	4.8–5.2	5.4	3.3	4.5	4.8
Bilirubin, total: mg/dl	0–3	0.3	0.3	0.3	0.2
Alkaline phosphatase, units/liter	207–335	306	588	339	248
ALT (SGPT), units/liter	69–139	195	106	36	90
AST (SGOT), units/liter	110–144	432	138	141	134
GGTP, units/liter	6.6–15.4	54	24	3	8
Cholesterol, mg/dl	89.4–106.6	156	99	144	142
Calcium, mg/dl	8.8–9.2	12	9.6	10.2	11
Phosphorus, mg/dl	3.2–6.8	5.7	3.9	3.9	4.4
Na, mEq/liter	148–150	160	144	160	144
K, mEq/liter	4.5–5.5	6	5.5	5.4	5.6
Cl, mEq/liter	98.3–111.7	108	97	90	102
BUN/Cr, ratio	16–26	18	20	25	14
Globulin, g/dl	2.6–3.4	3.6	3.6	3.9	3.8
Hemoglobin	15–19	15.5	17.6	ND	15.5
Hematocrit	46–58	46.4	51.2	ND	48.2
WBC	6.2–7.8	15.3	7	ND	7.1
RBC	6.4–7.6	5.82	7.07	ND	5.92
Bands	0	0	0	ND	0
Polymorphonuclear leukocytes	22–54	16	55	ND	26
Lymphocytes	38–72	82	42	ND	73
Monocytes	1.2–4.8	2	2	ND	1
Eosinophils	0.7–2.7	0	1	ND	0

Abbreviations: PI, Postinjection; ALT, alanine transaminase (SGPT, serum glutamic-pyruvic transaminase); AST, aspartate aminotransferase (SGOT, serum glutamic-oxaloacetic transaminase); GGTP, γ-glutamyltranspeptidase; BUN, blood urea nitrogen; Cr, creatinine; WBC, white blood cell; RBC, red blood cell; ND, not determined, due to clotting.

[a]Blood analysis of *Aotus nancymae* inoculated intraprostatically with G207 virus (1×10^7 PFU). Data shown are for day 7 (T348) and day 14 (T442, T635, and T659) postinoculation. Preoperation values are the average values of the four prostate-injected (T348, T635, T442, and T659) and two noninjected (T317 and T344) monkeys.

of HSV-induced histopathology or systemic dissemination in these hosts injected with G207. Therefore, in this study, 1×10^7 PFU of G207 was injected into the prostate of BALB/c mice and *Aotus* monkeys. This dose is efficacious in a number of tumor models, including glioma, neuroblastoma, etc. For human prostate tumor cells, we have previously reported that intratumoral inoculation using 2×10^7 PFU of G207 resulted in complete regression (cures) in approximately 22% (DU-145) to 25% (LNCaP) of tumors established in BALB/c *nu/nu* mice (Walker *et al.*, 1999).

Safety of intraprostatic G207 in *Aotus* and mice was assessed on the basis of animal survival, viral biodistribution, virus shedding, and histopathology. The survival data indicate that none of the injected mice or monkeys succumbed to G207. This was in contrast to strain F-injected mice, in which half died within 13 days postinjection despite the strain F dose being 1 log lower than the G207 dose. It is interesting to note that 50% of mice died after intraprostatic inoculation with 1×10^6 PFU of strain F as compared with 1×10^3 PFU after intracerebral inoculation or 1×10^4 PFU after intracerebroventricular inoculation (data from Sundaresan *et al.*, 2000).

No monkeys were injected intraprostatically with the parental strain F, but we have shown previously that these monkeys are exquisitely sensitive to HSV-1 strain F, with en-

FIG. 2. Representative photomicrographs of hematoxylin and eosin-stained histology sections of prostates from mock-treated mice (*top left*) or from G207 (*top right*)- or strain F (*bottom left* and *right*)-injected BALB/c mice. Mock and G207-injected prostates show normal architecture and histology whereas strain F-injected prostates show epithelial flattening and stromal edema (*left*), and epithelial sloughing (*right*). Original magnification: *top left*, ×60; *top right*, ×60; *bottom left*, ×120; *bottom right*, ×60.

FIG. 3. Representative photomicrographs of hematoxylin and eosin-stained histology sections of prostate from G207 (1×10^7 PFU)-injected (T635 and T442) or noninjected (T317 and T344) *Aotus nancymae*. G207-injected prostates show normal architecture and histology similar to those of the noninjected monkeys. Original magnification: T635 and T317, ×120; T442 and T344, ×60.

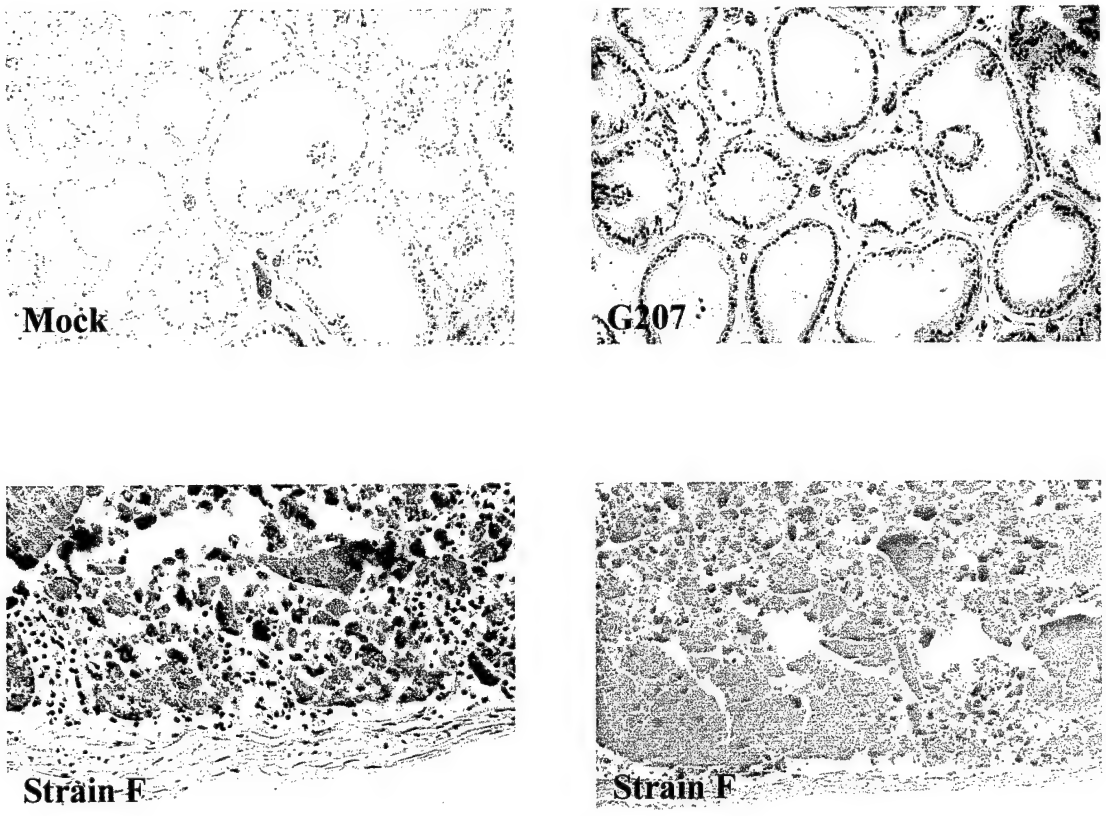

FIG. 2

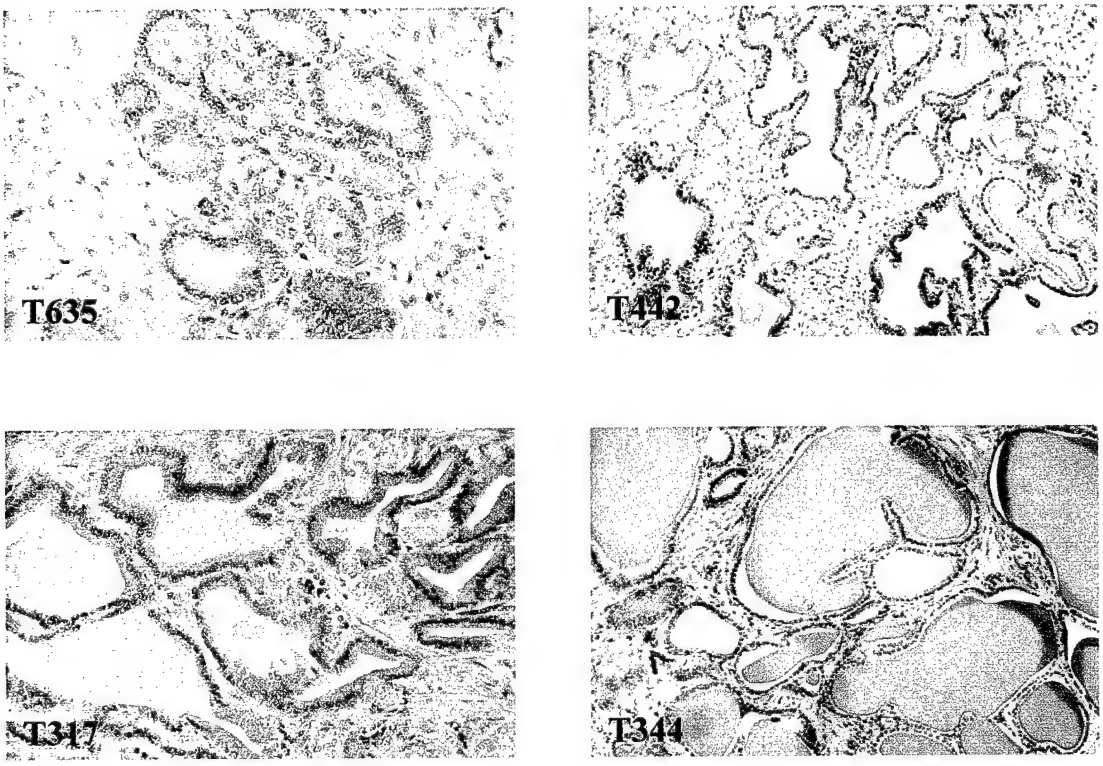

FIG. 3

TABLE 3. SERUM ANTI-HSV-1 ANTIBODY TITER IN *Aotus nancymae*[a]

	Short term		*Long term*	
	T348	*T635*	*T442*	*T659*
Preinjection (day −60)	2.36	0	2.56	0.01
Postinjection (sacrifice day)	1.58	ND	1.79	1.42

[a]Serum anti-HSV-1 antibody titer in *Aotus nancymae* before and after intraprostatic injection with G207 virus (1×10^7 PFU). Determination of titer was performed by enzyme-linked immunosorbent assay (ELISA). Values shown represent absorption at OD_{595}. ND, Not done.

cephalitis developing within 5 days of an intracerebral (1×10^3 PFU) inoculation (Hunter *et al.*, 1999). On the other hand, G207-injected monkeys in this study displayed no abnormalities on physical or neurological examination. Minor variations in blood chemistry values were observed and were considered insignificant to the study since none of these changes were consistently observed in all the injected animals. Some of these changes (abnormal glucose, ALT, AST, GGTP levels) were observed in the subjects even before prostate injection with G207 (data not shown). The *Aotus* monkeys used in this study were immunologically uncompromised on the basis of normal CBC values obtained prior to the study and no significant change was observed during the study. In response to G207 injection, the naive animal, T659, seroconverted for HSV-1 antibody and the preexposed (intracerebral) animals, T348 and T442, continued to generate anti-HSV-1 antibodies.

In ascertaining the safety of G207, which is a replication-competent virus, potential shedding of the virus was monitored by assaying for infectious virus in blood, CSF, urine, semen, and various mucosal (urethral, rectal, buccal, and conjunctival) sites. Extensive analysis on days 7, 14, and 21 for short-term monkeys and on days 14, 28, and 56 for long-term monkeys demonstrated the absence of any infectious virus. This observation is important in light of the finding that continuous virus shedding occurs throughout the period of survival in owl monkeys infected with wild-type HSV-1 (Meignier *et al.*, 1990). In addition, tissue distribution of the virus was assessed by PCR detection of HSV-1 DNA polymerase sequences from various tissues obtained at necropsy (day 14 or 21 for short-term monkeys, T348 and T635, and day 56 for long-term monkeys, T442 and T659). None of the monkeys tested had PCR-detectable sequences in prostate (except T659) or the neighboring tissues such as testis, adrenals, and urethra (except T659 with 66 copies) at the end of the study period, suggesting a lack of prolonged viral replication or latency in the normal urogenital tissues. A significant copy number was detected, however, in the brain and especially in the injection site (left frontal lobe) of the two monkeys (T348 and T442) that had been previously inoculated with G207. Since the two naive monkeys, T635 and T659, did not contain detectable HSV-1 sequences at this site, it would indicate that the origin of the viral sequences detected in T348 and T442 is from the prior intracerebral inoculation and is not due to dissemination from the prostate injection.

The lack of viral spread from prostate to distant tissues is further supported by the histopathological findings of various tissues obtained at necropsy in both mice and *Aotus* monkeys. All the G207-injected mice had normal prostate histology whereas the prostates from strain F-injected mice displayed varying degrees of epithelial sloughing and stromal edema. Most of the monkey tissues examined, including the prostates of T442 and T635, were found to have normal histology. All

TABLE 4. BIODISTRIBUTION ANALYSIS OF G207 INJECTED INTO THE PROSTATE OF *Aotus nancymae*[a,b]

	Short term		*Long term*	
	T348	*T635*	*T442*	*T659*
Prior intracerebral G207 inoculation:	*Yes*	*No*	*Yes*	*No*
Postinoculation day:	*Day 14*	*Day 21*	*Day 56*	*Day 56*
Temporal lobe	460	Neg.	NQ	Neg.
Frontal lobe (left)	140	NBR	1400	Neg.
Frontal lobe (right)	Neg.	NBR	14	Neg.
Spinal cord (cervical)	NQ	ND	ND	ND
Spinal cord (thoracic and lumbar)	ND	NBR	310	NBR
Retroperitoneal ganglia	NBR	Neg.	ND	NQ
Retroperitoneal lymph node	NQ	NQ	Neg.	12
Adrenals	Neg.	NQ	Neg.	NBR
Spleen	Neg.	Neg.	NQ	28
Testis	Neg.	Neg.	NBR	Neg.
Prostate	Neg.	Neg.	Neg.	NQ
Urethra (penile)	Neg.	NBR	Neg.	66

Abbreviations: Neg., Negative; NQ, not quantifiable (1–10 copies); NBR, not biologically relevant (less than 1 copy); ND, not done.

[a]Numbers represent copy number.

[b]Biodistribution analysis of G207 (1×10^7 PFU) injected into the prostate of *Aotus nancymae*, using a standardized real-time (TaqMan) PCR assay, for the detection of HSV-1 DNA polymerase sequences from total DNA from tissues collected at necropsy. The assay was consistently linear between 10 and 10^5 template copies and detects 100 copies of template in the presence of 1 μg of monkey genomic DNA.

TABLE 5. HISTOPATHOLOGY OF TISSUES FROM *Aotus nancymae*[a]

	Short-term injected		*Long-term injected*		*Prostate noninjected*	
Prior intracerebral G207 inoculation: Postinoculation day:	*T348 Yes Day 14*	*T635 No Day 21*	*T442 Yes Day 56*	*T659 No Day 56*	*T317 Yes*	*T344 Yes*
Brain	Normal	Normal	Normal	Normal	ND	ND
Spinal cord	Normal	Normal	Normal	ND	ND	ND
Retroperitoneal lymph node	Hemorrhage, mild	Hemorrhage, mild	Normal	Normal	NA	NA
Lung	Congestion, mild	Congestion, mild	Congestion, mild	Congestion, mild	Congestion, mild	Congestion, mild
Heart	ND	ND	ND	Normal	ND	ND
Liver	KC-pigmentation, Hepato-pigmentation, Centro-dissoc./degen.	KC-pigmentation, Hepato-pigmentation, Centro-dissoc./degen.	KC-pigmentation, Hepato-pigmentation, Centro-dissoc./degen.	KC-pigmentation, Hepato-pigmentation, Centro-dissoc./degen.	KC-pigmentation, Hepato-pigmentation, Centro-dissoc./degen.	KC-pigmentation, Hepato-pigmentation, Centro-dissoc./degen.
Spleen	ND	ND	ND	Normal	ND	ND
Pancreas	Islet cell hyperplasia, mild	ND	ND	Normal	Normal	Normal
Kidney	Inflammation, mild	Inflammation, mild	Inflammation, mild	Inflammation, mild	Inflammation, mild	Inflammation, mild
Adrenals	ND	Normal	Normal	Normal	ND	ND
Testis/vas deferens	Normal	Normal	Normal	Tubular degeneration	Normal	Normal
Prostate	NA	Normal	Normal	NA	Normal	Normal
Erectile tissue	Normal	Normal	Normal	NA	Normal	ND
Periprostatic lymph node	ND	Normal	ND	ND	ND	ND
Bladder	Normal	Hemorrhage, mild	Normal	ND	Normal	Normal
Rectum	Normal	Normal	Normal	ND	ND	ND
Pelvic fat	Normal	ND	Normal	ND	ND	ND
Bone marrow	ND	ND	ND	Normal	ND	ND

Abbreviations: ND, Not determined; KC, Kupffer cell; NA, not available; Centro-dissoc., centroacinar dissociation; degen., degeneration.

[a]Histopathology of tissues obtained at necropsy from *Aotus nancymae*. T348, T635, T442, and T659 were injected with G207 (1×10^7 PFU) intraprostatically; T317 and T344 were not injected intraprostatically. Tissues were fixed in formalin and paraffin embedded. Sections were stained with hematoxylin and eosin.

the *Aotus* monkeys exhibited changes in kidney, liver, and lung, which seemed unrelated to the study, as this characteristic was found to be common in this species (Todo *et al.*, 2000). Both of the prostate-noninjected monkeys, T317 and T344, also had these histological features. The absence of histological changes, even in tissues containing PCR-detectable viral DNA sequences, supports the safety of G207 injection into the prostate. Taken together, these results demonstrates that G207, a conditionally replication-competent virus, effective against human prostate tumor xenografts in mice, is a safe vector in both mice and nonhuman primates after intraprostate inoculation.

Whether immunosuppression, such as after chemotherapy, would alter the spread of virus is unknown. However, two earlier studies conducted with G207 may address this indirectly. We conducted a phase I clinical trial of G207 in patients with malignant glioma (Markert *et al.*, 2000). Some of these patients were receiving dexamethasone, an immunosuppressive drug, and no encephalitis or other HSV-1-associated disease was detected at doses up to 3×10^9 PFU of G207. In a mouse syngeneic tumor model, short-term administration of dexamethasone did not affect the efficacy of G207 treatment or the titer of infectious G207 isolated from tumors measured up to 5 days postinjection (Todo *et al.*, 1999b).

One concern about using G207 in treating human prostate cancer is whether reactivation of wild-type HSV-1 can occur from dorsal root ganglia and cause disease in individuals who are latently infected with HSV. Although this has not been tested directly after intraprostatic inoculation with G207, two studies suggest that reactivation might be unlikely. We have previously shown that superinfection with 1×10^7 PFU of G207 into the brains of BALB/c mice in which prior KOS strain infection was established did not lead to detectable reactivation of infectious virus or disease (Sundaresan *et al.*, 2000). Also, Wang *et al.* (1997) have shown that intracerebral infection with hrR3 strain of HSV-1 did not reactivate wild-type KOS virus in adult rats that were latently infected with KOS by corneal scarification or intracerebral injection.

Paielli *et al.* (2000) reported on the safety evaluation of a replication-competent adenovirus (Ad5-CD/TK*rep*) administered intraprostatically in C57BL/6 mice and showed that the virus persisted in the mouse urogenital tract (prostate and testis) and liver up to 28 days postinjection but with minimal toxicity (Paielli *et al.*, 2000). Virus persistence in the male urogenital tract was not associated with germ line transmission of the virus. However, persistence in the liver was associated with sustained but minimal inflammation of liver and gall bladder, which became acute with prodrug (5-fluorouracil plus ganciclovir) therapy. In a phase I clinical trial, administration of replication-deficient adenovirus (ADV/RSV-tk) into the prostate of humans resulted in some hepatic toxicity after ganciclovir (GCV) treatment (Herman *et al.*, 1999). The results from our study, demonstrating that intraprostatic administration of G207 does not lead to any complications in the liver of mice and nonhuman primates, suggest that use of G207 might prove to be a safe alternative for viral and/or gene therapy of prostate cancer. In particular, it might be initially appropriate for locally recurrent prostate cancer after radiation therapy since surgery has a higher complication rate and a higher incidence of neural damage in those patients who were previously irradiated. G207 has been shown to be efficacious in treating human prostate cancer recurring in animal models after radiation therapy (Walker *et al.*, 1999). In addition, G207 was found also to act in some settings as an *in situ* cancer vaccine by inducing systemic antitumor immunity (Toda *et al.*, 1999; Todo *et al.*, 1999a).

The issue of whether G207 can replicate in normal human prostate tissue is not conclusively addressed in this study and destruction of normal prostate tissue by G207 could potentially occur. Nevertheless, the destruction of normal prostate tissue would likely be clinically tolerated, as it is different from surgical removal, so long as the tumor is destroyed and the adjoining nervous tissue is spared. Most of the side effects, such as incontinence or erectile dysfunction, observed with current treatment protocols for prostate cancer, arise from nerve tissue damage. This can be mitigated with the use of G207, which is nonpathogenic to nerves, and thus offers an advantage for the treatment of prostate cancer. The current study demonstrating safety of intraprostatically injected G207 in mice and nonhuman primates, and previous data of G207 safety in murine peripheral nerves and in the brain of humans, provide a strong basis for a phase I trial of G207 for prostate cancer as a "nerve-sparing" vector.

ACKNOWLEDGMENTS

This study was supported by grants to R.L.M. from the Association for the Cure of Cancer of the Prostate (CapCure) and the United States Department of Defense (Army Prostate Cancer Research Program) and from NeuroVir, Inc. We also acknowledge the contributions of Jonathan Walker, M.D., and Ali Sajadi, M.D. (residents of the Department of Urology, Georgetown University Hospital, Washington, D.C.), and Laura Rutter-Call (Veterinary Technician, Division of Comparative Medicine, Georgetown University Medical Center, Washington, D.C.). S.D.R. and R.L.M. are consultants to NeuroVir, Inc., which has a license from Georgetown University to commercialize G207.

REFERENCES

ANDREANSKY, S.S., HE, B., GILLESPIE, G.Y., SOROCEANU, L., MARKERT, J., CHOU, J., ROIZMAN, B., and WHITLEY, R.J. (1996). The application of genetically engineered herpes simplex viruses to the treatment of experimental brain tumors. Proc. Natl. Acad. Sci. U.S.A. **93,** 11313–11318.

BENNETT, J.J., KOOBY, D.A., DELMAN, K., McAULIFFE, P., HALTERMAN, M.W., FEDEROFF, H., and FONG, Y. (2000). Antitumor efficacy of regional oncolytic viral therapy for peritoneally disseminated cancer. J. Mol. Med. **78,** 166–174.

BOVIATSIS, E.J., CHASE, M., WEI, M.X., TAMIYA, T., HURFORD, R.K., JR., KOWALL, N.W., TEPPER, R.I., BREAKEFIELD, X.O., and CHIOCCA, E.A. (1994). Gene transfer into experimental brain tumors mediated by adenovirus, herpes simplex virus, and retrovirus vectors. Hum. Gene Ther. **5,** 183–191.

BRANDT, C.R., IMESCH, P.D., ROBINSON, N.L., SYED, N.A., UNTAWALE, S., DARJATMOKO, S.R., CHAPPELL, R.J., HEINZELMAN, P., and ALBERT, D.M. (1997). Treatment of spontaneously arising retinoblastoma tumors in transgenic mice with an attenuated herpes simplex virus mutant. Virology **229,** 283–291.

CHAHLAVI, A., TODO, T., MARTUZA, R.L., and RABKIN, S.D.

(1999). Replication-competent herpes simplex virus vector G207 and cisplatin combination therapy for head and neck squamous cell carcinoma. Neoplasia **1,** 162–169.

COUKOS, G., MAKRIGIANNAKIS, A., MONTAS, S., KAISER, L.R., TOYOZUMI, T., BENJAMIN, I., ALBELDA, S.M., RUBIN, S.C., and MOLNAR-KIMBER, K.L. (2000). Multi-attenuated herpes simplex virus-1 mutant G207 exerts cytotoxicity against epithelial ovarian cancer but not normal mesothelium and is suitable for intraperitoneal oncolytic therapy. Cancer Gene Ther. **7,** 275–283.

DORKIN, T.J., and NEAL, D.E. (1997). Basic science aspects of prostate cancer. Semin. Cancer Biol. **8,** 21–27.

EASTHAM, J.A., CHEN, S.H., SEHGAL, I., YANG, G., TIMME, T.L., HALL, S.J., WOO, S.L., and THOMPSON, T.C. (1996). Prostate cancer gene therapy: Herpes simplex virus thymidine kinase gene transduction followed by ganciclovir in mouse and human prostate cancer models. Hum. Gene Ther. **7,** 515–523.

HERMAN, J.R., ADLER, H.L., AGUILAR-CORDOVA, E., ROJAS-MARTINEZ, A., WOO, S., TIMME, T.L., WHEELER, T.M., THOMPSON, T.C., and SCARDINO, P.T. (1999). In situ gene therapy for adenocarcinoma of the prostate: A phase I clinical trial. Hum. Gene Ther. **10,** 1239–1249.

HOLM, H.H., JUUL, N., PEDERSEN, J.F., HANSEN, H., and STROYER, I. (1983). Transperineal 125iodine seed implantation in prostatic cancer guided by transrectal ultrasonography. J. Urol. **130,** 283–286.

HUNTER, W.D., MARTUZA, R.L., FEIGENBAUM, F., TODO, T., MINETA, T., YAZAKI, T., TODA, M., NEWSOME, J.T., PLATENBERG, R.C., MANZ, H.J., and RABKIN, S.D. (1999). Attenuated, replication-competent herpes simplex virus type 1 mutant G207: Safety evaluation of intracerebral injection in nonhuman primates. J. Virol. **73,** 6319–6326.

JORGENSEN, T.J., KATZ, S., WITTMACK, E.K., VARGHESE, S., TODO, T., RABKIN, S., and MARTUZA, R. (2001). Ionizing radiation does not alter the antitumor activity of herpes simplex virus vector G207 in subcutaneous tumor models of human and murine prostate cancer. Submitted.

KO, S.C., GOTOH, A., THALMANN, G.N., ZHAU, H.E., JOHNSTON, D.A., ZHANG, W.W., KAO, C., and CHUNG, L.W. (1996). Molecular therapy with recombinant p53 adenovirus in an androgen-independent, metastatic human prostate cancer model. Hum. Gene Ther. **7,** 1683–1691.

KRAMM, C.M., CHASE, M., HERRLINGER, U., JACOBS, A., PECHAN, P.A., RAINOV, N.G., SENA-ESTEVES, M., AGHI, M., BARNETT, F.H., CHIOCCA, E.A., and BREAKEFIELD, X.O. (1997). Therapeutic efficiency and safety of a second-generation replication-conditional HSV1 vector for brain tumor gene therapy. Hum. Gene Ther. **8,** 2057–2068.

LAMBRIGHT, E.S., CAPARRELLI, D.J., ABBAS, A.E., TOYOIZUMI, T., COUKOS, G., MOLNAR-KIMBER, K.L., and KAISER, L.R. (1999). Oncolytic therapy using a mutant type-1 herpes simplex virus and the role of the immune system. Ann. Thorac. Surg. **68,** 1756–1760. [Discussion on pp. 1761–1752]

LEE, J.H., FEDEROFF, H.J., and SCHOENIGER, L.O. (1999). G207, modified herpes simplex virus type 1, kills human pancreatic cancer cells in vitro. J. Gastrointest. Surg. **3,** 127–131.

LU, Y., CARRAHER, J., ZHANG, Y., ARMSTRONG, J., LERNER, J., ROGERS, W.P., and STEINER, M.S. (1999). Delivery of adenoviral vectors to the prostate for gene therapy. Cancer Gene Ther. **6,** 64–72.

MARKERT, J.M., MEDLOCK, M.D., RABKIN, S.D., GILLESPIE, G.Y., TODO, T., HUNTER, W.D., PALMER, C.A., FEIGENBAUM, F., TORNATORE, C., TUFARO, F., and MARTUZA, R.L. (2000). Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: Results of a phase I trial. Gene Ther. **7,** 867–874.

MARTUZA, R.L., MALICK, A., MARKERT, J.M., RUFFNER, K.L., and COEN, D.M. (1991). Experimental therapy of human glioma by means of a genetically engineered virus mutant. Science **252,** 854–856.

MASHOUR, G.A., MOULDING, H.D., CHAHLAVI, A., KHAN, G.A., RABKIN, S.D., MARTUZA, R.L., HERNAIZ-DRIEVER, P., and KURTZ, A. (2001). Therapeutic efficacy of G207 in a novel peripheral nerve sheath tumor model. Exp. Neurol. **169,** 64–71.

McKIE, E.A., BROWN, S.M., MACLEAN, A.R., and GRAHAM, D.I. (1998). Histopathological responses in the CNS following inoculation with a non-neurovirulent mutant (1716) of herpes simplex virus type 1 (HSV 1): Relevance for gene and cancer therapy. Neuropathol. Appl. Neurobiol. **24,** 367–372.

MEIGNIER, B., MARTIN, B., WHITLEY, R.J., and ROIZMAN, B. (1990). In vivo behavior of genetically engineered herpes simplex viruses R7017 and R7020. II. Studies in immunocompetent and immunosuppressed owl monkeys (*Aotus trivirgatus*). J. Infect. Dis. **162,** 313–321.

MINETA, T., RABKIN, S.D., and MARTUZA, R.L. (1994). Treatment of malignant gliomas using ganciclovir-hypersensitive, ribonucleotide reductase-deficient herpes simplex viral mutant. Cancer Res. **54,** 3963–3966.

MINETA, T., RABKIN, S.D., YAZAKI, T., HUNTER, W.D., and MARTUZA, R.L. (1995). Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. Nat. Med. **1,** 938–943.

MIYATAKE, S., IYER, A., MARTUZA, R.L., and RABKIN, S.D. (1997). Transcriptional targeting of herpes simplex virus for cell-specific replication. J. Virol. **71,** 5124–5132.

NASU, Y., BANGMA, C.H., HULL, G.W., LEE, H.M., HU, J., WANG, J., McCURDY, M.A., SHIMURA, S., YANG, G., TIMME, T.L., and THOMPSON, T.C. (1999). Adenovirus-mediated interleukin-12 gene therapy for prostate cancer: Suppression of orthotopic tumor growth and pre-established lung metastases in an orthotopic model. Gene Ther. **6,** 338–349.

OYAMA, M., OHIGASHI, T., HOSHI, M., NAKASHIMA, J., TACHIBANA, M., MURAI, M., UYEMURA, K., and YAZAKI, T. (2000). Intravesical and intravenous therapy of human bladder cancer by the herpes vector G207. Hum. Gene Ther. **11,** 1683–1693.

PAIELLI, D.L., WING, M.S., ROGULSKI, K.R., GILBERT, J.D., KOLOZSVARY, A., KIM, J.H., HUGHES, J., SCHNELL, M., THOMPSON, T., and FREYTAG, S.O. (2000). Evaluation of the biodistribution, persistence, toxicity, and potential of germ-line transmission of a replication-competent human adenovirus following intraprostatic administration in the mouse. Mol. Ther. **1,** 263–274.

PENG, K.W., and RUSSELL, S.J. (1999). Viral vector targeting. Curr. Opin. Biotechnol. **10,** 454–457.

POTOSKY, A.L., MILLER, B.A., ALBERTSEN, P.C., and KRAMER, B.S. (1995). The role of increasing detection in the rising incidence of prostate cancer. JAMA **273,** 548–552.

PYLES, R.B., WARNICK, R.E., CHALK, C.L., SZANTI, B.E., and PARYSEK, L.M. (1997). A novel multiply-mutated HSV-1 strain for the treatment of human brain tumors. Hum. Gene Ther. **8,** 533–544.

RANDAZZO, B.P., BHAT, M.G., KESARI, S., FRASER, N.W., and BROWN, S.M. (1997). Treatment of experimental subcutaneous human melanoma with a replication-restricted herpes simplex virus mutant. J. Invest. Dermatol. **108,** 933–937.

RODRIGUEZ, R., SCHUUR, E.R., LIM, H.Y., HENDERSON, G.A., SIMONS, J.W., and HENDERSON, D.R. (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: A selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. Cancer Res. **57,** 2559–2563.

SUNDARESAN, P., HUNTER, W.D., MARTUZA, R.L., and RABKIN, S.D. (2000). Attenuated, replication-competent herpes simplex virus type 1 mutant G207: Safety evaluation in mice. J. Virol. **74,** 3832–3841.

TODA, M., RABKIN, S.D., and MARTUZA, R.L. (1998). Treatment

of human breast cancer in a brain metastatic model by G207, a replication-competent multimutated herpes simplex virus 1. Hum. Gene Ther. **9,** 2177–2185.

TODA, M., RABKIN, S.D., KOJIMA, H., and MARTUZA, R.L. (1999). Herpes simplex virus as an in situ cancer vaccine for the induction of specific anti-tumor immunity. Hum. Gene Ther. **10,** 385–393.

TODO, T., RABKIN, S.D., SUNDARESAN, P., WU, A., MEEHAN, K.R., HERSCOWITZ, H.B., and MARTUZA, R.L. (1999a). Systemic antitumor immunity in experimental brain tumor therapy using a multimutated, replication-competent herpes simplex virus. Hum. Gene Ther. **10,** 2741–2755.

TODO, T., RABKIN, S.D., CHAHLAVI, A., and MARTUZA, R.L. (1999b). Corticosteroid administration does not affect viral oncolytic activity, but inhibits antitumor immunity in replication-competent herpes simplex virus tumor therapy. Hum. Gene Ther. **10,** 2869–2878.

TODO, T., FEIGENBAUM, F., RABKIN, S.D., LAKEMAN, F., NEWSOME, J.T., JOHNSON, P.A., MITCHELL, E., BELLIVEAU, D., OSTROVE, J.M., and MARTUZA, R.L. (2000). Viral shedding and biodistribution of G207, a multimutated, conditionally replicating herpes simplex virus type 1, after intracerebral inoculation in *Aotus*. Mol. Ther. **2,** 588–595.

TOYOIZUMI, T., MICK, R., ABBAS, A.E., KANG, E.H., KAISER, L.R., and MOLNAR-KIMBER, K.L. (1999). Combined therapy with chemotherapeutic agents and herpes simplex virus type 1 ICP34.5 mutant (HSV-1716) in human non-small cell lung cancer. Hum. Gene Ther. **10,** 3013–3029.

WALKER, J.R., McGEAGH, K.G., SUNDARESAN, P., JORGENSEN, T.J., RABKIN, S.D., and MARTUZA, R.L. (1999). Local and systemic therapy of human prostate adenocarcinoma with the conditioning replicating herpes simplex virus vector G207. Hum. Gene Ther. **10,** 2237–2243.

WANG, Q., GUO, J., and JIA, W. (1997). Intracerebral recombinant HSV-1 vector does not reactivate latent HSV-1. Gene Ther. **4,** 1300–1304.

WOOLF, S.H. (1995). Screening for prostate cancer with prostate-specific antigen. An examination of the evidence. N. Engl. J. Med. **333,** 1401–1405.

YAZAKI, T., MANZ, H.J., RABKIN, S.D., and MARTUZA, R.L. (1995). Treatment of human malignant meningiomas by G207, a replication-competent multimutated herpes simplex virus 1. Cancer Res. **55,** 4752–4756.

YOON, S.S., CARROLL, N.M., CHIOCCA, E.A., and TANABE, K.K. (1998). Cancer gene therapy using a replication-competent herpes simplex virus type 1 vector. Ann. Surg. **228,** 366–374.

YU, D.C., CHEN, Y., SENG, M., DILLEY, J., and HENDERSON, D.R. (1999a). The addition of adenovirus type 5 region E3 enables calydon virus 787 to eliminate distant prostate tumor xenografts. Cancer Res. **59,** 4200–4203.

YU, D.C., SAKAMOTO, G.T., and HENDERSON, D.R. (1999b). Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. Cancer Res. **59,** 1498–1504.

Address reprint requests to:
Dr. Robert L. Martuza
Neurosurgical Service, Department of Surgery
Massachusetts General Hospital
WHT-502, 55 Fruit Street
Boston, MA 02114

E-mail: rmartuza@partners.org

Received for publication December 15, 2000; accepted after revision March 16, 2001.

Ionizing Radiation Does Not Alter the Antitumor Activity of Herpes Simplex Virus Vector G207 in Subcutaneous Tumor Models of Human and Murine Prostate Cancer

Timothy J. Jorgensen[1,3,*]
Sanford Katz[1]
Ellen K. Wittmack[1]
Susan Varghese[2,4]
Tomoki Todo[2,4]
Samuel D. Rabkin[2,4]
Robert L. Martuza[2,4]

[1]Department of Radiation Medicine
[2]Department of Neurosurgery
[3]Department of Biochemistry and Molecular Biology

Lombardi Cancer Center
Georgetown University Medical Center
Washington, D.C. 20007

RUNNING TITLE: Radiation and viral therapy for prostate cancer
KEY WORDS: Herpes virus, gene therapy, prostate cancer, radiotherapy

*Corresponding author:

Timothy J. Jorgensen, Ph.D.
Department of Radiation Medicine
Georgetown University Medical Center
Lombardi Cancer Center/TRB room E212A
3970 Reservoir Road, N.W.
Washington, D.C. 20007-2197

telephone 202-687-1810
facsimile 202-687-2221
e-mail jorgensent@odrge.odr.georgetown.edu

[4]Current address: Molecular Neurosurgery Laboratory, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129

ABSTRACT

Viral gene therapy against malignant tumors holds great promise for tumors that are susceptible to the oncolytic activity of viruses. One advantage of oncolytic viral therapy is that it can potentially be combined with other therapies, such as radiotherapy, to obtain an enhanced tumor response. In the case of prostate cancer, herpes simplex virus (HSV) mediated therapies have been shown to be highly effective in animal models; however, studies of the efficacy of combined viral and radiation therapy have not yet been reported. In this study, we have combined G207, a multimutated HSV type 1 vector, with external beam radiation therapy of prostate tumors grown subcutaneously in mice. We examined both the human LNCaP tumor in athymic mice, and the mouse transgenic TRAMP tumor in either athymic mice or its syngeneic host, C57BL/6 mice. Virus was delivered either intravenously, in the case of LNCaP, or intratumorally, in the case of TRAMP. We found that individually, either G207 or radiation was effective in delaying tumor growth in these models. However, delivering the treatments simultaneously did not produce an enhanced effect.

NONSTANDARD ABBREVIATIONS: None

INTRODUCTION

The use of replication-competent viruses for tumor therapy is a promising strategy that has progressed to early clinical trials (1-5). Replication-competent or conditionally-replicating herpes simplex virus type 1 (HSV-1) vectors have been generated by mutating genes involved in viral DNA synthesis and/or virulence in order to target viral replication and toxicity to tumor cells (6, 7). G207 is a multimutated HSV-1 vector that lacks both copies of the ICP34.5 gene, a gene required for neurovirulence, and contains an insertion of the lacZ gene inactivating the ICP6 gene, encoding the large subunit of ribonucleotide reductase (8). Studies in both human xenograft and syngeneic mouse tumor models have demonstrated the value of HSV based therapies in terms of both growth inhibition and cures (9). These findings await confirmation in human clinical trials.

One advantage of viral gene therapy is that it can potentially be combined with other therapies to obtain an enhanced tumor response. In fact, recent reports about combination viral/radiotherapy and viral/chemotherapy in glioma and head and neck cancer animal models suggest that this may be a viable approach (10-15). Human prostate tumor cells are particularly sensitivity to HSV-1 vectors (16), and this has lead to experimental treatment strategies that deliver mutated viruses by either intratumoral or intravenous injection. However, studies on the efficacy of combined viral and radiotherapy have not been reported. In this study, we assess the ability of radiation to affect the activity of G207 against prostate cancer. G207 treatment was combined with external beam radiation therapy of prostate cancer grown subcutaneously in mice. We used both the LNCaP human tumor in athymic mice, and the transgenic TRAMP mouse

tumor in either athymic mice or its syngeneic host, C57BL/6 mice. Virus was delivered either intravenously, in the case of LNCaP, or intratumorally, in the case of TRAMP. We found that G207 and radiation were each effective in producing growth delay in these models, but simultaneously delivering the treatments did not produce an enhanced effect.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The human prostate cancer cell line LNCaP (Georgetown University Medical Center, Lombardi Cancer Center Tissue Culture Shared Resource, Washington, DC) was maintained in RPMI 1640 (Biofluids) containing 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). The TRAMP-C2 mouse prostate cancer cell line (17) was grown in DMEM high glucose (DMEM-HG, Life Technologies, Inc.) supplemented with 5% fetal bovine serum (Life Technologies, Inc.), 5% Nu-serum IV (Collaborative Biomedical Products), and 10^{-8} M dihydrotestosterone (Sigma Chemical Co., St Louis, MO). Penicillin-streptomycin (Mediatech) and L-glutamine (Mediatech) were added to each culture and the cells were maintained at 37°C with 5% CO_2. Both cell lines were confirmed to be free of mycoplasma contamination.

Preparation and Injection of Cells into Animals

Four to six week old C57BL/6 black or NCRNU athymic male mice were obtained from Harlan Laboratories (Indianapolis, IN), or Taconic Laboratories (Germantown, NY), respectively. All animals were quarantined for one week before the study and allowed access to food and water *ad libitum*. The animals were anesthetized by intraperitoneal injection of 0.15-0.2 ml of 10% sodium pentobarbital (Abbott Laboratories, North Chicago, IL) in bacteriostatic saline (0.9% sodium chloride; Abbott Laboratories) with

6% ethyl alcohol. C57BL/6 mice were shaved in the rump before injections. $1x10^7$ cells in 0.1 ml were injected subcutaneously in the sacral region of each animal to induce tumors in the TRAMP experiments. For the LNCaP experiments, the cells ($1x10^7$) were first mixed with an equal volume of Matrigel (Collaborative Biochemical Products, Bedford, MA) and then injected. Tumors were measured twice weekly by calipers to within 0.5 mm, and volumes were calculated (V=HxLxW) and recorded. Animals were randomized into treatment groups once their tumor size was in the range of 100-320 mm^3. After tumor cell injection, LNCaP tumors took 5-6 weeks to grow to treatment size, whereas TRAMP tumors grew in 1-3 weeks. In the LNCaP experiment, animals were given G207 (2×10^7 pfu) by tail vein injection on days 0 and 4, and irradiated on days 1-5. In both TRAMP experiments, animals were given G207 (2×10^7 pfu) intratumorally on days 0, 3, and 6, and irradiated daily on days 1-5. On days with both virus injection and irradiation, the virus was given to the animals before the irradiation occurred. The animal procedures described here were approved by the Georgetown University Animal Care and Use Committee.

Irradiation of Prostate Tumors LNCaP and TRAMP

A ^{137}Cs Shepherd Mark I irradiator was used to irradiate the tumors in the sacral region of the animal. The mice were restrained in clear plastic holders with a lead cover, which contains a port through which the radiation can enter to irradiate the tumor. The holders were placed behind a lead wall, which shields the mouse's body, exposing the

irradiation port of the holder above the edge of the wall. Four animals were irradiated simultaneously. Doses to the tumor under this geometry were confirmed using a phantom mouse and thermoluminescent dosimetry. In the LNCaP experiment, the tumors were given 10 Gy fractionated over days 1-5 (i.e. 2 Gy/day). In the TRAMP experiments, the tumors were given a total of 20 Gy over days 1-5 (i.e. 4 Gy/day).

Clonogenic Cell Survival Curves

Logarithmically growing cells were harvested and seeded into tissue culture flasks at various cell numbers depending upon the radiation dose which the flask was to receive (i.e. more cells for higher doses), so that the final number of survivors in each flask would be similar. After allowing time for attachment, the flasks were irradiated to various doses and returned to the incubator for two weeks. The flasks were stained to reveal colonies produced from the clones of surviving cells and counted. The fraction of survivors relative to the original number of cells seeded was calculated and then normalized to the zero-dose plating efficiency to determine the surviving fraction at each dose. The data were fitted to the single-hit multitarget curve model (18).

RESULTS

Human LNCaP Tumor Treatment

LNCaP is a commonly used hormone-responsive human prostate cell line that grows well, albeit slowly, as subcutaneous tumors in athymic mice (19). LNCaP tumor

cells are particularly sensitive to G207, both *in vitro* and *in vivo* (16). The LNCaP tumor is wild-type for p53 and secretes prostate specific antigen (PSA) (19). *Its cellular radioresponses have been characterized for both clonogenic survival and apoptosis* (20-23). Because the LNCaP/athymic mouse xenograft model has been prevalent and important in prostate cancer research (24), we chose this as one of our models for studying combined radiation/G207 effects.

We previously found LNCaP tumors to be highly sensitive to intratumorally and intravenously injected G207 (16). Even a single intratumoral injection of G207 (2×10^7 pfu) caused a major reduction in tumor volumes with complete eradication of 25% of the tumors. This viral response was too great for combined radiation/viral studies, where partial responses for each agent are needed to assess potential interactions. Even when the viral titres were lowered to 10^5 pfu significant cures ensued (data not shown). For this reason, we decided to employ intravenous treatment with G207, which we knew gave a less robust treatment response (16). We employed two intravenous injections spread over four days, and combined it with five daily fractions of radiation, starting on the day after the first viral treatment.

Radiation therapy was effective at inhibiting tumor growth (**Fig. 1**), however, the irradiated tumors grew back relatively quickly. In contrast, volumes of G207-treated tumors were markedly reduced and regrowth occurred much more slowly than for irradiated tumors (**Fig. 1**). Nevertheless, by 40 days all of the tumors had started to grow back. For the combined radiation and G207 treatment, tumors regressed slightly faster than for G207 alone, but ultimately reached the same minimum volume -- again on day 40

-- at which point they began to regrow at a rate that was indistinguishable from G207 treatment alone (**Fig 1**). Nadir tumor volumes were statistically significantly different at $p < 0.05$ between all treatment groups, except for radiation plus G207 versus G207 alone, which were not significantly different from each other. There were no cures in any of the groups.

Murine TRAMP Tumor Treatment

TRAMP represents a relatively new animal model for prostate cancer research (17, 25). In this syngeneic mouse model, transgenic mice carry the SV40 large tumor antigen linked to a prostate specific promoter. Expression of the transgene in prostate tissue caused tumors to arise *in situ* at about 8 weeks of age, and these tumors resembled naturally arising prostate cancer. Several tumor cell lines have been established in tissue culture from the prostate tumors of TRAMP mice. These tumor lines no longer express large T antigen (17).

TRAMP tumor cell lines grow very well subcutaneously in either athymic mice or in the syngeneic parental mice, C57BL/6, from which the TRAMP transgenic mice were derived. This feature of the model provided major advantages, since G207 treatment of murine tumors in syngeneic mice leads to a potent antitumor immune response (26, 27). The TRAMP tumor model allows us to examine the effect of the immune system on both treatment strategies and their combination by comparing efficacy in immunocompromised and immunocompetent mice.

The TRAMP model system is not as well characterized for radiation responses as LNCaP; however, it is known to be wild-type for p53, like LNCaP (17). We were able to confirm the p53 phenotype by showing radiation induction of the p53-transcriptionally-activated $p21^{WAF1/CIP1}$ protein in both LNCaP and TRAMP cells in vitro (data not shown). Preliminary studies with subcutaneous TRAMP tumors suggested that they were twice as resistant to radiation therapy compared to LNCaP. This was consistent with *in vitro* clonogenic cell survival curve analysis (**Fig. 2**), suggesting that the tumor resistance of TRAMP was an intrinsic property of the cells, and not due to possible tumor physiology differences. Hypoxia, for example, has been reported to affect tumor radioresponses in other prostate cancer animal models (28). Due to the greater radioresistance of TRAMP tumors, 20 Gy was used for the therapy dose, rather than the 10 Gy that was used for LNCaP.

Mouse tumor cells, in general, are less susceptible to G207 replication and cytotoxicity than human tumor cells. Growth curves for untreated TRAMP tumors in athymic mice showed that they grow twice as fast as LNCaP tumors, and the delay produced by G207 was much less than that for LNCaP (**Fig. 3**). TRAMP tumors treated with radiation therapy alone produced about twice the growth delay of G207 alone. Combined radiation and G207 produced no greater delay than radiation therapy alone.

In order to assess the possible influence of an intact immune system in the TRAMP response to G207 and radiation, the TRAMP tumor experiment was also conducted in C57BL/6 mice (**Fig. 4**). Compared to the athymic mouse host, G207 was much more effective in inhibiting TRAMP tumor growth in C57BL/6 mice (**Fig. 3** vs.

Fig. 4). In fact, the G207 delay was now greater than the delay for radiation alone. This increased efficacy of G207 in syngeneic mouse model is consistent with results obtained with CT26 colon carcinoma in BALB/c mice, N18 neuroblastoma in A/J mice, and M3 melanoma in DBA/2 mice (26, 27), suggesting that immune responses contribute to the antitumor activity of G207.

In contrast to the G207 response, tumor growth with radiation alone was similar in athymic and C57BL/6 mice, suggesting that the immune system was not affecting TRAMP tumor regression caused by radiotherapy. Although tumor radioresponses are largely thought to be driven by intrinsic properties of tumors(29), recently it has been reported that T-cells and natural killer cells may promote radiotherapy tumor regression in some tumors(30). Nevertheless, in our model the presence or absence of a thymus did not affect tumor radioresponse, and the combined radiation and G207 treatment produced no greater tumor delay than G207 therapy alone, in either host. Also, an interaction between G207 and radiation treatment was not seen for the TRAMP tumor regardless of the host strain.

DISCUSSION

It has been reported that the efficacy of therapeutic HSV-1 R3616 against subcutaneous and intracranial human U87 malignant glioma xenografts in athymic mice is significantly enhanced by radiation (10, 13). Similar findings were reported for HSV-1 R7020 against subcutaneous human head and neck cancer cell line, SQ20b (11). In the current study, G207, another therapeutic herpes vector is evaluated for combination

therapy against prostate cancer. *When the combination of G207 and radiation did not enhance efficacy in the TRAMP syngeneic tumor model, we hypothesized that this might be due to species differences or the effect of cellular immunity. Therefore, we examined a human xenograft in athymic mice, as was the case in the Weichselbaum studies (10, 11, 13).* In neither a human nor a mouse tumor model system was there a benefit from combining radiation with G207.

In our experiments, radiation treatments were fractionated over five consecutive days (i.e. experimental days 1-5). We find that this fractionation regimen is amenable to a wide variety of subcutaneous tumor models, regardless of differences in tumor growth rates or intrinsic cellular radiosensitivities, and it allows easier cross comparisons between the radioresponses of tumors since the number and timing of fractions is always constant. For intratumoral injections, we used three injections, timed to be before (day 0), during (day 3), and after (day 6) radiation therapy, in order to help ensure that we would cover all possible temporal sensitivity windows for radiation/virus interactions. For intraveneous injections we injected on day 0 and day 4, because we had previously shown that this injection regimen worked well with LNCaP tumors(16). *It was not our intention to directly duplicate the protocols Weichselbaum's group because different experimental regimens were used in each of their three publications*(10, 11, 13), *and no particular protocol appeared to be critical. Also, because radiation treatments with different doses and fractionation schemes was seen to enhance the antitumor activity of both R7020 and R3616 in glioma and carcinoma, we did not expect that it would be necessary to exactly imitate a particular treatment regimen in order to see an effect with*

G207. Furthermore, Weichselbaum and coworkers did not directly compare the effect of different time delays of irradiation post-infection; however, in the one study where different delays were used, there were no indications that a shorter time (4 hr) was better than a longer delay (24 hr). Therefore, we do not believe that the failure of radiation and G207 to act synergistically or additively in our prostate tumor models can be attributed merely to minor temporal differences between virus and irradiation treatments. Rather, it is more likely that differences in tumor type and biology play a role in the phenomenon reported by Weichselbaum and coworkers.

Despite the fact that the combination of G207 and radiation did not enhance the tumor response over the most effective individual therapy, there are several findings that are important for designing tumor therapy. The TRAMP tumor maintained the same radiosensitivity in either the athymic or immunocompetent host. This was expected, since immune responses are not thought to be a major factor in tumor radioresponses (30). G207, however, worked worse than radiation in athymic mice, but better than radiation in C57BL/6 mice. This illustrates the dual factors contributing to the viral antitumor response -- direct oncolytic activity and induction of tumor-specific immune responses.

Although radiation did not sensitize the tumors to virus, it also did not decrease the efficacy of the virus either. These results combined with our earlier published results (16), indicating that tumors that recurred following radiation therapy remained sensitive to virus, suggest that G207 therapy might be a useful therapy for tumors that recur in the radiation field. Also, since localized radiotherapy would not result in systemic immunosuppression, it is less likely than chemotherapy to interfere with subsequent

immune-mediated tumor cell killing by G207. Along these lines, intravenous administration of herpes virus following irradiation might have the added benefit of targeting metastases, that local radiation therapy alone cannot reach. Conversely, these results suggest that little benefit is derived from delivering both treatments simultaneously. Since the radiation adds little to the cytotoxicity of prostate tumors produced by the virus alone, delivering radiation at the time of viral treatment might even preclude later use of radiotherapy to treat recurring local disease. *These results taken together with our earlier findings that recurring irradiated tumors remained fully sensitive to G207* (16) *suggest that sequential, rather than simultaneous, treatment with radiation followed by G207 may produce the best results in terms of delaying tumor regrowth and prolonging survival.*

The discrepancy between these findings in prostate, and those previously reported with glioma and head and neck cancer, may reflect more than simple tumor type differences. R3613 and R7020 are more virulent viruses that replicate better than G207. If, as the previous investigators suggest, the enhanced effect is due to increased viral replication in irradiated cells (10) -- possibly as the result of host cell DNA damage (31) -- then viruses with a high replication capacity might be better able to capitalize on this. Alternatively, the relatively high sensitivity of prostate tumors to G207 herpes virus (16) may mean that they are already fully sensitized to the cytotoxic effects of the virus, and irradiation cannot further enhance killing by virus. Regardless, our findings suggest that tumor sensitization to herpes viral therapy by radiation is not a universal phenomenon,

and that both viral and tissue specific factors may be strong determinants of the effect, and warrant further study.

ACKNOWLEDGEMENTS

This study was supported by grants from the Association for the Cure of Cancer of the Prostate (CaP Cure) and the United States Department of Defense DAMD17-98-1-8490 (Army Prostate Cancer Research Program). S.D.R. and R.L.M. are consultants to MediGene Inc., which has a license from Georgetown University for G207.

REFERENCES

1. Markert JM, Medlock MD, Rabkin SD, Gillespie GY, Todo T, Hunter WD, Palmer CA, Feigenbaum F, Tornatore C, Tufaro F, and Martuza RL (2000). Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. *Gene Ther* 7, 867-874.

2. Rampling R, Cruickshank G, Papanastassiou V, Nicoll J, Hadley D, Brennan D, Petty R, MacLean A, Harland J, McKie E, Mabbs R, and Brown M (2000). Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma [see comments]. *Gene Ther* 7, 859-866.

3. Khuri FR, Nemunaitis J, Ganly I, Arseneau J, Tannock IF, Romel L, Gore M, Ironside J, MacDougall RH, Heise C, Randlev B, Gillenwater AM, Bruso P, Kaye SB, Hong WK, and Kirn DH (2000). A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat Med* 6, 879-885.

4. Ganly I, Eckhardt SG, Rodriguez GI, Soutar DS, Otto R, Robertson AG, Park O, Gulley ML, Heise C, Von Hoff DD, and Kaye SB (2000). A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer [published erratum appears in Clin Cancer Res 2000 May;6(5):2120]. *Clin Cancer Res* 6, 798-806.

5. Lamont JP, Nemunaitis J, Kuhn JA, Landers SA, and McCarty TM (2000). A prospective phase II trial of ONYX-015 adenovirus and chemotherapy in recurrent squamous cell carcinoma of the head and neck (the Baylor experience) [In Process Citation]. *Ann Surg Oncol* 7, 588-592.

6. Jacobs A, Breakefield XO, and Fraefel C (1999). HSV-1-based vectors for gene therapy of neurological diseases and brain tumors: Part II. Vector systems and applications. *Neoplasia* 1, 402-416.

7. Rabkin SD, Mineta T, Miyatake S, and Yazaki T (1996). Gene therapy: targeting tumor cells for destruction. *Hum Cell* 9, 265-276.

8. Mineta T, Rabkin SD, Yazaki T, Hunter WD, and Martuza RL (1995). Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nature Medicine* 1, 938-943.

9. Martuza RL (2000). Conditionally replicating herpes vectors for cancer therapy. *J Clin Invest* 105, 841-846.

10. Advani SJ, Sibley GS, Song PY, Hallahan DE, Kataoka Y, Roizman B, and Weichselbaum RR (1998). Enhancement of replication of genetically engineered herpes simplex viruses by ionizing radiation: a new paradigm for destruction of therapeutically intractable tumors. *Gene Therapy* 5, 160-165.

11. Advani SJ, Chung SM, Yan SY, Gillespie GY, Markert JM, Whitley RJ, Roizman B, and Weichselbaum RR (1999). Replication-competent, nonneuroinvasive genetically engineered herpes virus is highly effective in the treatment of therapy-resistant experimental human tumors. *Cancer Res* 59, 2055-2058.

12. Chahlavi A, Todo T, Martuza RL, and Rabkin SD (1999). Replication-competent herpes simplex virus vector G207 and cisplatin combination therapy for head and neck squamous cell carcinoma. *Neoplasia* 1, 162-169.

13. Bradley JD, Kataoka Y, Advani S, Chung SM, Arani RB, Gillespie GY, Whitley RJ, Markert JM, Roizman B, and Weichselbaum RR (1999). Ionizing radiation improves survival in mice bearing intracranial high-grade gliomas injected with genetically modified herpes simplex virus. *Clin Cancer Res* 5, 1517-1522.

14. Markert JM, Gillespie GY, Weichselbaum RR, Roizman B, and Whitley RJ (2000). Genetically engineered HSV in the treatment of glioma: a review. *Rev.Med.Virol.* 10, 17-30.

15. Chmura SJ, Advani SJ, Kufe DW, and Weichselbaum RR (1999). Strategies for enhancing viral-based gene therapy using ionizing radiation. *Radiat.Oncol.Investig.* 7, 261-269.

16. Walker JR, McGeagh KG, Sundaresan P, Jorgensen TJ, Rabkin SD, and Martuza RL (1999). Local and systemic therapy of human prostate adenocarcinoma with the conditionally replicating herpes simplex virus vector G207. *Hum.Gene Ther.* 10, 2237-2243.

17. Foster BA, Gingrich JR, Kwon ED, Madias C, and Greenberg NM (1997). Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. *Cancer Res* 57, 3325-3330.

18. Albright N (1987). Computer programs for the analysis of cellular survival data. *Radiation Research* 112, 331-340.

19. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, and Murphy GP (1983). LNCaP model of human prostatic carcinoma. *Cancer Res* 43, 1809-1818.

20. Kimura K, Bowen C, Spiegel S, and Gelmann EP (1999). Tumor necrosis factor-alpha sensitizes prostate cancer cells to gamma-irradiation-induced apoptosis. *Cancer Res* 59, 1606-1614.

21. Garzotto M, Haimovitz-Friedman A, Liao WC, White-Jones M, Huryk R, Heston WD, Cardon-Cardo C, Kolesnick R, and Fuks Z (1999). Reversal of radiation resistance in LNCaP cells by targeting apoptosis through ceramide synthase. *Cancer Res* 59, 5194-5201.

22. Nava VE, Cuvillier O, Edsall LC, Kimura K, Milstien S, Gelmann EP, and Spiegel S (2000). Sphingosine enhances apoptosis of radiation-resistant prostate cancer cells. *Cancer Research* 60, 4468-4474.

23. Colletier PJ, Ashoori F, Cowen D, Meyn RE, Tofilon P, Meistrich ME, and Pollack A (2000). Adenoviral-mediated p53 transgene expression sensitizes both wild-type and null p53 prostate cancer cells *in vitro* to radiation. *Int.J.Radiat.Oncol.Biol.Phys.* 48, 1507-1512.

24. Navone NM, Logotheis CJ, Von Eschenbach AC, and Troncoso P (1999). Model sysyems of prostate cancer: uses and limitations. *Cancer and Metastasis Reviews* 17, 361-371.

25. Greenberg NM, DeMayo F, Finegold MJ, Medina D, Tilley WD, Aspinall JO, Cunha GR, Donjacour AA, Matusik RJ, and Rosen JM (1995). Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci U S A* 92, 3439-3443.

26. Todo T, Rabkin SD, Sundaresan P, Wu A, Meehan KR, Herscowitz HB, and Martuza RL (1999). Systemic antitumor immunity in experimental brain tumor therapy using a multimutated, replication-competent herpes simplex virus. *Hum Gene Ther* 10, 2741-2755.

27. Todo M, Rabkin SD, Kojima H, and Martuza RL (1999). Herpes simplex virus as an in situ cancer vaccine for the induction of specific anti-tumor immunity. *Hum Gene Ther* 10, 385-393.

28. Leith JT, Quaranto L, Padfield G, Michelson S, and Hercbergs A (1993). Radiobiological studies of PC-3 and DU-145 human prostate cancer cells: X-ray sensitivity *in vitro* and hypoxic fractions of xenografted tumors *in vivo*. *Int.J.Radiat.Oncol.Biol.Phys.* 25, 283-287.

29. Steel GG, Adams GE, and Horwich A, Eds. *The biological basis of radiotherapy.* Elsevier, Amsterdam.

30. Mason K, Staab A, Hunter N, McBride W, Petersen S, Terry N, and Milas L (2001). Enhancement of tumor radioresponse by docetaxel: Involvement of immune system. *Int J Oncol* 18, 599-606.

31. Miller CS, and Smith KO (1991). Enhanced replication of herpes simplex virus type 1 in human cells. *Journal of Dental Research* 70, 111-117.

LEGENDS

Figure 1: Mean relative volumes of LNCaP prostate tumors in athymic male mice. The mean relative tumor volumes, compared to the first day of treatment, of tumor-bearing mice treated with virus buffer (Controls), G207, irradiation (RT), or both (RT + G207). Irradiated animals were treated for Days 1-5 of the experiment with 2-Gy daily fractions for a total dose of 10 Gy. Virus-treated animals were given virus at 2×10^7 pfu by tail vein injection on Days 0 and 4 of the experiment. Each point represents the mean tumor volume plus/minus 1 S.E.M. In some cases where individual animals were sacrificed due to large tumor burden, the tumor size at the time of sacrifice was used for subsequent calculations of mean tumor volume.

Figure 2: Radiation survival curves for TRAMP and LNCaP cells. Points represent the mean of four independent experiments, each performed on different days; bars represent the standard deviation of the means. The data were fitted to the single-hit multitarget survival model and D_0 and D_q parameters, respectively, measured in Gy, for each cell line which were as follows: Tramp, 1.763 and 1.219; LNCaP, 1.015 and 1.631.

Figure 3: Mean relative volumes of TRAMP prostate tumors in athymic male mice. The mean relative tumor volumes, compared to the first day of treatment, of tumor-bearing mice treated with virus buffer (Controls), G207, irradiation (RT), or both (RT + G207). Irradiated animals were treated for Days 1-5 of the experiment with 4-Gy daily

fractions for a total dose of 20 Gy. Virus-treated animals were given virus at 2 x 10^7 pfu by intratumoral injections on Days 0, 3, and 6 of the experiment. Each point represents the mean tumor volume plus/minus 1 S.E.M. In some cases where individual animals were sacrificed due to large tumor burden, the tumor size at the time of sacrifice was used for subsequent calculations of mean tumor volume.

Figure 4: Mean relative volumes of TRAMP prostate tumors in male C57BL/6 mice. The mean relative tumor volumes, compared to the first day of treatment, of tumor-bearing mice treated with virus buffer (Controls), G207, irradiation (RT), or both (RT + G207). Irradiated animals were treated for Days 1-5 of the experiment with 4-Gy daily fractions for a total dose of 20 Gy. Virus-treated animals were given virus at 2 x 10^7 pfu by intratumoral injections on Days 0, 3 and 6 of the experiment. Each point represents the mean tumor volume plus/minus 1 S.E.M. In some cases where individual animals were sacrificed due to large tumor burden, the tumor size at the time of sacrifice was used for subsequent calculations of mean tumor volume.